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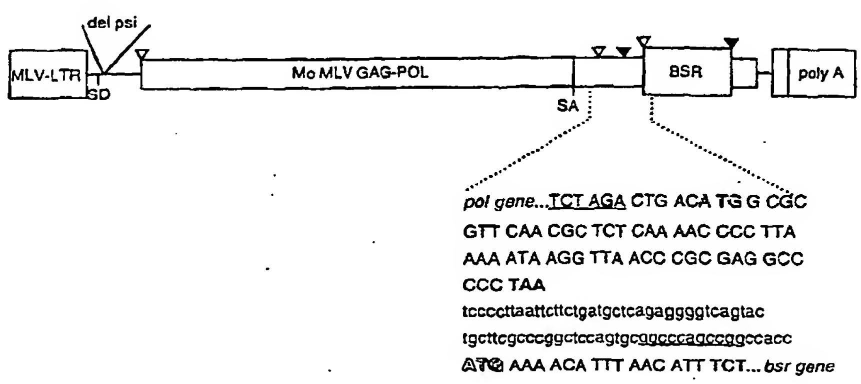

Schematic structure of CeB expression vector

(57) Abstract

The invention relates to new expression systems and in particular to an expression system in which a gene of interest is expressed at an optimal level. The invention provides a recombinant expression vector comprising a gene of interest and a selectable marker gene, wherein the selectable marker gene is arranged downstream of the gene of interest and a stop codon associated with the gene of interest is spaced from a start codon of said selectable marker gene at a distance which is sufficient to ensure that translation reinitiation is required before said selectable marker protein is expressed from the corresponding MRNA. Examples of such expression systems are vector viral packaging cell lines and a number of preferred cell lines have been identified.

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Expression systems

The present invention relates to new expressions systems, and in particular to expression systems in which a gene of interest is expressed at an optimal level. Particular examples of such expression systems are retroviral packaging cell lines and a number of preferred cell lines have been identified.

The ability of eukaryotic and prokaryotic ribosomes to reinitiate translation at an internal start codon within an mRNA sequence has previously been recognised. Studies have been reported in which the efficiency of the process, which is generally regarded as being low, has been connected with the length of the intercistronic sequence (Kozak (1987) Mol. Cell Biol. 7, 3438-3445). Selection of this sequence or spacer as 70bp in length, and containing no other start codons, has been previously reported as being optimal for reinitiation in a eukaryotic cell line (Cosset F-L., Virology (1991) 185, 862).

The applicants have found a way in which the inefficiency associated with the translation reinitiation process can be used to good effect.

According to the present invention there is provided a recombinant expression vector comprising a gene of interest and a selectable marker gene, wherein the selectable marker gene is arranged downstream of the gene of interest and a stop codon associated with the gene of interest is spaced from a start codon of said selectable marker gene at a distance which is sufficient to ensure that translation re-initiation is required before said selectable marker protein is expressed from the corresponding mRNA.

The invention further provides a process for producing cell lines in which a gene of interest is expressed, which process comprises transforming host cells with an expression vector comprising said gene of interest and a selectable marker gene, wherein the selectable marker gene is arranged downstream of the gene of interest and a stop codon associated with the gene of interest is spaced from a start codon of said selectable marker gene at a distance which is sufficient to ensure that translation re-initiation is required before said selectable marker protein is expressed from the corresponding mRNA, and selecting those cells where expression of the selectable marker gene may be detected.

Since re-initiation of translation is a relatively inefficient process, this means that the selectable marker protein will be expressed at lower levels than the product of the gene of interest. When the marker protein is expressed at detectable levels, the gene of interest will be expressed at higher levels. This will ensure that during the subsequent selection procedure, only those cell clones which express the gene of interest at higher or optimal levels will survive. Low expressing clones will be eliminated by the selection process.

Cells transformed with the above-described expression vectors form a further aspect of the invention.

The host cells are suitably eukaryotic or prokaryotic host cells, preferably eukaryotic host cells.

The number of nucleotides in the space between the stop codon of the gene of interest and the start codon of the selectable marker will suitably be in the range of from 20-200 nucleotides, preferably from 60-80 nucleotides, even more preferably 70-80 nucleotides.

The vectors used in the process of the invention may be any of the known types, for example expression plasmids or viral vectors.

Selected cells may be cultured and if required, the protein product of the gene of interest isolated from the culture using conventional techniques. Alternatively, expression of the gene of interest may result in other desired effects, for example, where the gene of interest is included as part of a viral packaging construct.

Some experimental and clinical gene transfer protocols require the design of gene transfer vectors suitable for *in vivo* gene delivery (**Miller, A.D.** 1992. Nature 357:455-460). Retroviral vectors are attractive candidates for such applications, because they can provide stable gene transfer and expression (Samarut J. et al.,Meth. Enzymol. in press) and because packaging cells have been designed which produce non-replication competent viruses (Miller A.D (1990) Hum Gene Ther. 1 5-14). However currently available recombinant retroviruses suffer from a number of drawbacks.

Packaging cell lines provide <u>in trans</u> the retroviral proteins encoded by the <u>gag</u>, <u>pol</u>, and <u>env</u> genes required to obtain infectious retroviral particles. The <u>gag</u> and <u>pol</u> products are respectively the structural components of the virion cores and the replication machinery (enzymes) of the retroviral particles whereas the <u>env</u> products are envelope proteins responsible for the host-range of the virions and for the initiation of infection and for sensitivity to humoral factors. An ideal packaging cell line should produce retroviruses that only contain the retroviral vector genome, and absolutely no replication-competent genomes or defective genomes encoding some of the viral structural genes.

A number of packaging cell lines designed for human gene transfer have been designed in the past by introducing plasmid DNAs which contain "helper genomes" encoding gag, pol and/or env genes into cells.

Retroviral packaging cell lines are cells that have been engineered to provide in trans all the functions required to express infectious retroviral vectors. A helper genome (or construct or unit), is herein also referred to as "retroviral packaging construct (or unit)" or "packaging-deficient construct (or genome unit)" or "gag-pol/env expression plasmids".

Much efforts has been made to design strategies to optimize the helper-genomes in order (i) to get the highest production of retroviral packaging functions (which correlates which infection titers of retroviral particles) and (ii) to minimise the chance that the helper genome can be transmitted via the viral particles (which may lead to emergence of unwanted retroviral forms).

The first of these packaging cell lines used full length retroviral genomes as helper genomes that had been crippled for important cis-regulated replicative functions (reviewed in Miller, Hum. Gene. Ther. 1:5-14 1990). In order to reduce the possibility of occurrence of replication-competent viruses and of transfer of virus structural genes, a second generation of safer packaging cell lines has been designed by using two separate and complementary helper genomes which express either gag-pol or env and are packaging-deficient (Miller supra).

The cells into which these helper genomes were introduced were isolated by cotransfecting them with plasmids encoding selectable markers. However, as no selection was applied on

the packaging-deficient retroviral genome itself, the helper functions can be lost during the passages of the cells in culture and the current packaging systems provide limited titers of infectious retroviral vectors, usually only of the order of 10^5-10^6 infectious units i.u/ml. Indeed the cotransfection with a plasmid encoding a selectable marker does not directly select the best gag-pol-env-expressing cells.

The invention further provides a retroviral packaging cell line comprising a host cell transformed with (i) a packaging deficient construct which expresses a viral gag-pol gene and a first selectable marker gene, and/or (ii) a packaging-deficient construct which expresses a viral env gene and a second selectable marker gene; wherein a start codon of the first and second selectable markers are spaced from the stop codons of the viral gag-pol gene and the viral env gene respectively by a distance which ensures that reinitiation of mRNA translation is required for expression of marker protein product of said first and/or second selectable marker gene.

The retroviral packaging cell line may be obtained by the above described process which will involve selecting transfected cells which express said first and/or second marker genes.

By using helper constructs which are directly selectable and which provide for high expression of the viral gene, high titre retroviral vectors may be obtained.

Helper constructs for use in the process form a furtner aspect of the invention.

The retroviral vectors prepared from the conventional

packaging cell lines are usually not contaminated by replication-competent retroviruses (RCRs). However, recombinant amphotropic murine retroviruses have been shown to arise spontaneously from certain packaging cell lines. The generation of such RCRs involves recombination at least between gag-pol/env packaging sequence and vector sequences (Cosset et al., Virology, (1993) 193:385-395).

Recombinant RCRs have been associated with the development of lymphomas in some severely immunosuppressed monkeys (Donahue et al., J. Exp Med (1992) 176: 1125-1135). In addition, retroviral vector preparations may also contain, at low frequencies, retroviruses coding for functional envelope glycoproteins (Kozak and Kabat, 1990, J. Virol. 64: 3500-3508) or for gag-pol proteins. Although the pathogenicity of these gag-pol or env recombinant retroviruses is probably low, more evolved recombinant retroviruses with higher pathogenic potential may occur when injected in vivo, by recombination and/or complementation of the initial recombinant viruses with some endogenous retroviruses.

In a preferred embodiment of the retroviral packaging cell lines of the invention, the overlapping sequences between the genomes of the retroviral vector and the helper construct are reduced, for example as compared to constructs such as CRIPenv and CRIPAMgag (Danos et al., Proc. Natl. Acad. Sci USA 85: 6460-6464). In particular, the viral sequences in the helper construct are reduced, for example, not only the packaging sequence but also the 3' Long Terminal Repeat (LTR), the 3' non-coding sequence and/or the 5'LTR may be eliminated.

The possibility of generation of such RCRs and recombinant retroviruses can be reduced by reducing the overlapping

sequences between the genomes of both the retroviral vector and the helper construct.

Conventional retroviral vectors are strongly inactivated by human serum which makes them of limited or no use for *in situ* gene transfer in gene therapy applications. It has previously been shown that inactivation by complement in human serum is controlled by the cell line used to produce the virions and by viral envelope determinants (Takeuchi et al., J. Virol (1994) 68:8001-8007). In particular, inactivation is caused by some properties of the cell lines that have been used to construct the packaging cells (NIH-3T3) and also by viral determinants located in the retroviral envelope as shown (Takeuchi et al., J. Virol (1994) 68:8001-8007). *In vivo* gene delivery is an important goal for a number of human gene therapy strategies.

The applicants have found that certain cell lines form preferred packaging cell lines.

Particularly preferred packaging cell lines are the HT1080 line, the TE671 line, the 3T3 line, the 293 line and the Mv-1-Lu line. One example of retroviral packaging cells that will produce complement-resistant virus comprise human HT1080 cells and express RD114 envelope. Such cells form a preferred aspect of the invention.

Packaging cell lines according to the invention provide 50-100 fold increased titers of retroviral vectors as compared to conventional packaging cell lines. Retroviral vectors provided by these new cells are safe, in terms of generation of RCRs, and considerably more resistant to inactivation by human complement.

Packaging cell lines according to the invention may be able

to transduce helper-free, human complement-resistant retroviral vectors at titers consistently higher than 10^7 i.u./ml.

Suitable semi-packaging cell lines in accordance with the invention are those which express only the gag-pol genes. Such cell lines may suitably be derived from TE671, MINK Mv-1-Lu, HT1080, 293 or NIH-3T3 cells by introduction of plasmid CeB (the MoMLV gag-pol expression unit).

Particularly preferred expression vectors in accordance with the invention for use in retroviral packaging cell lines are those which include MLV gag and pol genes such as CeB. Other plasmids may include gag and pol genes from other retroviruses or chimeric or mutated gag and pol genes.

Various viral and retroviral envelope genes may be included in the plasmids such as MLV-A envelope, GALV envelope, VSV-G protein, BaEV envelope, RD114 envelope and chimeric or mutated envelopes. Plasmids which include the RD114 env gene such as FBdelPRDSAF as illustrated hereinafter, provide one example of suitable constructs.

The novel retroviral packaging cells described hereinafter, have been designated FLY cells, and may be designed for in vivo gene delivery.

Considerable variations were found between the various cell lines screened for their ability to release type C mammalian retroviruses. In addition, few cell lines were able to produce retroviruses completely resistant to human complement. Based on these two criteria, human fibrosarcoma HT1080 and rhabdomyosarcoma TE671 cells were selected for optimum construction of packaging cells.

Other studies have shown the importance of endogenous retrovirus expression in the generation of recombinant retroviruses from retroviral packaging lines (Ronfort et al., Virology, (1995), 207, 271-275, Vanin, E.F.et al., J Virol (1994) 68:4241-4250.). The co-packaging of an endogenous genome and a vector can lead to emergence of recombinant retroviruses (Vanin et al., supra). Recombination involves template switching during reverse transcription of such hybrid retroviruses (Hu et al., Science, (1990) 250:1227) and homologies between the two genomes considerably enhance the frequency of reverse transcriptase jumps (Zhang et al., J. Virol. (1994) 68: 2409-2414). Therefore an ideal packaging cell line should not express endogenous MLV-like (or type C retrovirus-like) retroviral genomes which can be packaged by type C gag proteins (Scadden et al., J. Virol. (1990) 64: 424-427, Torrent et al., J. Mol. Biol. (1994) 240 434-444).

Packaging of human endogenous retroviral RNA was not detected in TELCeB and FLY packaging cells when virion associated RNA was analysed by RT-PCR using generic primers. HT1080- and TE671 derived packaging cell lines may be safer in this respect than those generated from NIH3T3 cells, such as GP+EAM12 cells, which are known to express and package sequences related to type C retroviruses (Scadden et al. supra).

To generate the FLY packaging cell lines, HT1080 cells were transfected with gag-pol and env expression plasmids designed to optimise viral protein expression. Direct selection for viral gene expression was achieved in accordance with the invention by expression of a selectable marker gene by re-initiation of translation of the mRNA expressing the viral proteins. This strategy resulted in packaging cell lines capable of producing extremely high

titer viruses. Furthermore, long-term expression of packaging functions can be maintained in these cells. Many unnecessary viral sequences were eliminated from the packaging constructs to reduce the risk of helper virus generation; indeed the final packaging cells did not produce helper virus, in that no replication competent virus (RCR) could be detected per 10^7 vector particles.

The FLY packaging cells described herein are safer than, for example, psiCRIP cells, at least for generation of env recombinant retroviruses as is illustrated in Table 4 hereinafter, probably because less retroviral sequences overlapping with the vector were present in the present env-expression plasmid. Few reports have addressed the question of the characterization of recombinant retroviruses (RVs) (**Cosset, F.L., et al.**, Virology (1993) 193:385-395). It is possible that such RVs could not be detected in previous packaging cell lines due to lower overall titers. RVs are defective in normal cell culture conditions but are likely to evolve to replication competent viruses if they are allowed to replicate in cells complementing their expression like co-cultivated packaging cells (Bestwick et al., Proc. Natl Acad Sci USA, (1988) 85: 5404-5408, Cosset et al., (1993) supra).

In preferred retroviral packaging systems according to the invention, RVs are eradicated for example by removal of viral LTRs from the packaging construct.

Consistent with our previous studies (**Takeuchi, Y., et al.**, J Virol (1994) 68:8001-8007), LacZ(RD114) and lacZ(MLV-A) pseudotypes produced from HT1080 and TE671cells were more resistant to human complement than LacZ(RD114) or LacZ(MLV-A) pseudotypes produced by 3T3 of dog cells. It was therefore decided to use RD114 and MLV-A env genes to

generate recombinant virions with MoMLV cores.

The sequence of RD114 env gene was determined and is shown in Figure 4. It was found to be very close to BaEV (baboon endogenous virus) a type C retrovirus (**Benveniste, R.E.et al.**, Proc. Natl. Acad. Sci. USA (1973) 70:3316-3320; **Kato, S.et al.**, Japan. J. Genet. (**1987**) 62:127-137) with an envelope gene displaying similarities to the external part of type D simian retroviruses (SRVs). RD114 uses the SRV receptor on human cells (**Sommerfelt & Weiss**, Virology (1990) 176:58-69; **Sommerfelt, M.A. et al.**, J Virol (1990) 64:6214-6220) making the FLY packaging cells with RD114 envelope capable of generating virions with different tropism. Retroviral vectors prepared so far for human gene therapy have used either MLV-A or GALV (gibbon ape leukemia virus) envelopes which display some similarities (**Battini, J.L.,et al.**, J Virol. (1992) 66:1468-1475) and which use two related cell surface receptors for infection (**Miller, D.G. et al.**, J Virol (1994) 68:8270-8276). Differences in tissue-specific expression of MLV-A or GALV receptors have been reported (**Kavanaugh et al.**, Proc Natl Acad Sci USA (1994) 91:7071-7075).

The invention will now be particularly described by way of example with reference to the accompanying drawings in which:

Figure 1.illustrates the structure and expression of CeB. The env gene (Xbal-Clal) of plasmid pCRIP was removed and was replaced by coinsertion of the two fragments Xbal-Sfil (restriction sites underlined) from pOXEnv and a Sfil-Clal PCR product containing the bsr selectable marker. This results in positioning the bsr start codon (shadowed) 74 bp downstream to the pol stop codon (bold).

Open triangle are start codons (gag and bsr), black triangles are stop codons (pol and bsr). The shadowed triangle is the start codon of env, in the same reading frame with that of bsr. SD and SA are the splice donnor and splice acceptor sites.

Figure 2 illustrates the structure and expression of FbdelPASAF.
Immediately after the stop codon of env (bold) was inserted a non retroviral Kasl-Ncol (restriction sites underlined) linker which positions the phleo start codon (shadowed) 76 bp downstream.
Open triangle are start codons (env and phleo), black triangles are stop codons (env and phleo). SD and SA are the splice donnor and splice acceptor sites.

Figure 3 illustrates plasmids for expression of Ampho, Eco, RD114, Xeno, 10A1, GALV, VSV-G and FeLVB envelopes.
All genes are expressed in the same backbone as detailed in fig. 2. The BglII sites for ecotropic (MoMLV strain), 10A1, xenotropic (NZB.1.V6 strain) and amphotropic (4070A strain), the Ndel site of RD114 (SC3C strain, the BamHl site for both FeLVB and GALV were used as 5′ ends, and linked to Mscl site immediately after the splice donor site in the leader of FB29 LTR.

Figure 4 shows the sequence of the RD114 env gene (SEQ ID No 1).

Figure 5 shows the genetic structure of gag-pol constructs. Initiation (▽) and termination (▼) codons are shown. The thick dotted line below each construct shows MLV-derived sequences. Nucleotide positions of MLV-derived sequences are shown according to: Shinnick et al. (1981) (from nt 1 to nt 6000 with deletion of the packaging signal (DY) from BalI

(nt 215) to PstI (nt 568), and with some further MoMLV sequences in both CeB and CeB DS- from nt 7676 to nt 7938. gag-pol and bsr genes were expressed from the same transcription unit using the either a retroviral promoter (Mo LTR) or a non retroviral promoter (hCMV) and non retroviral polyadenylation sequence (polyA). Splice donor (SD) and acceptor (SA) sites are indicated. The thin line denotes retroviral non coding sequences. The thick line shows the rabbit beta-1 globin intron B. The position of some restriction sites is indicated.

The nucleic acid sequences of portions of constructs (as shown in **Figure** 5 (boxed areas)) are displayed for CeB (**SEQ ID No** 2, **Figure** 6), hCMV+intron (**SEQ ID No** 3, **Figure** 7) and hCMV+intronkaSD (**SEQ ID No** 4, **Figure** 8).

The nucleic acid sequences of portions of constructs (as shown in **Figure** 3 (boxed areas)) are displayed for FbdelPASAF (**SEQ ID No** 5, **Figure** 9), FbdelPMOSAF (**SEQ ID No** 6, **Figure** 10), FbdelPGASAF (**SEQ ID No** 7, **Figure** 11), FbdelPRDSAF (**SEQ ID No8**, **Figure** 12) and CMV10A1 (**SEQ ID No** 9, **Figure** 13) are shown.

The components of the viral particles are produced by two independent expression plasmids (<u>gag-pol</u> or <u>env</u>) which also contain selectable markers (<u>bsr</u> or <u>phleo</u>) expressed from the same transcriptional units as gag-pol or env (figs. 1& 2). The selectable markers are located downstream to <u>gag-pol</u> or env genes and there is an optimal distance between the stop codon of the upstream reading frames and the start codon of the selectable genes that should allow re-initiation of translation (Kozak, Mol Cell Biol. (1987) 7,:3438-3445). Because there is no "Kozak" sequence (Kozak, Cell,(1986) 44: 283-292) required for a normal initiation of translation for

the marker gene, they can only be expressed by re-initiation of translation after the upstream viral gene has been successfully expressed. Consequently and also because re-initiation of translation is a poorly efficient process, after transfection of these plasmids, cells resistant to the drugs corresponding to those selectable genes express high levels of the viral proteins.

To avoid viral transmission of these "helper" genomes the constructs used suitably have the classical deletions of both the packaging sequence located in the leader region and of the 3'LTR, the latter being replaced by SV40 polyadenylation sequences (Figs 1 & 2).

Plasmid CeB is the MoMLV gag-pol-expression unit. It derives from pCRIP, a plasmid used to generate the constructs introduced in the CRIP and CRE packaging cell lines (Danos and Mulligan, 1988). As shown in fig. 1 for generation of plasmid CeB the env gene of pCRIP has been deleted mostly and the bsr selectable marker, -encoding a protein conferring resistance to blasticidin (Izumi et al., Experimental Cell Research (1991) 197, 229-233)- has been inserted downstream to pol gene. There are exactly 74 bp with no ATG triplets between the stop codon of pol and the start codon of bsr, this allows its expression by re-initiation of translation on the gag-pol mRNA, after translation of the gag-pol reading frame.

FbdelPASAF is a plasmid expressing the amphotropic env gene and the phleo selectable marker conferring resistance to phleomycin (Gatignol et al., FEBS Letters (1988) 230:171-175). By using a PCR-mediated mutagenesis strategy which modifies the end of env gene (see fig. 2), a 76 bp linker was inserted between the stop codon of env and the start codon of phleo. This allows expression of phleo from the

env mRNA by re-initiation of translation. In addition compared to known env-expressing constructs, this strategy of construction has reduced the length of sequences overlapping with the ends of conventional retroviral vectors. The env genes of Mo-MLV, FeLVB, NZB.1V6, 10A1, GALV and RD114 are expressed by plasmids FBdelPMoSAF, FBdelPBSAF, FBdelXSAF, FBdelpGSAF, FBdelp10A1SALF and FBdelPRDSAF, respectively, by using the same backbone as FBdelPASAF (fig. 3). Retroviral vectors produced with the RD114 envelope will be useful for in vivo gene delivery as comparatively to MLV ecotropic or amphotropic envelopes, virions pseudotyped with RD114 envelopes are not inactivated by human complement when they are produced by Mink Mv-1-Lu cells or by some human cells (Table 1).

The HT1080 cell line, isolated from a human fibrosarcoma (ATCC CCL121). The TE671 cell line isolated from a human rhabdomyosarcoma (ATCC CRL 8805) (purchased from ATCC, and tested for absence of usual cell culture contaminants by ECACC), has been used for the definitive construction of packaging cell lines. HT1080 line was chosen among a panel of primate and human lines because MLV-A and RD114 efficiently rescued retroviral vectors from these cells and also because RD114 pseudotypes produced by this cell line were stable when incubated in human serum. In a standard assay (Takeuchi et al., J Virol (1994), 68, 8001-8007), these latter viruses were found more than 500 fold more stable than similar pseudotypes produced in 3T3 cells.

Another advantage for the use of non murine cells to derive packaging lines is the absence of MLV-related endogenous retroviral-like sequences (like VL30 in 3T3 cells) that can cross-package with MLV-derived retroviral vectors (Torrent et al., 1994) and generate potentially harmful recombinant retroviruses.

The helper constructs were introduced into other cell lines (HT1080 (table 2) Mink Mv-1-Lu (table 2), 3T3 (not shown), TE671 (table 2)) for the purpose of comparisons of the efficiency of the constructs.

As illustrated hereinafter (Table 2), the reverse transcriptase (RT) activity (provided by expression of the pol gene) in cells transfected with CeB is significantly higher than that of the same cells transfected by the parental plasmid pCRIP or that of cells chronically infected by MLV. This enhancement of viral gene expression is correlated with the titers of lacZ retroviral vectors when an envelope is provided in CeB-lacZ cells after comparison with titers of lacZ pseudotypes of either replication-competent viruses or other helper-free packaging systems.

For the generation of final packaging cell lines, the best clonal env transfectants have been selected. Packaging systems obtained in this way will be able to produce helper-free retroviral vectors at titers greater than 10^8 infectious particles per ml, which would be 10-100 fold higher to helper-free preparations of others.

Because of the way the selectable markers are expressed (see above), growing the packaging cells in phleomycin and blasticidin selective pressure increase and stabilize the expression of the retroviral components and particularly the envelopes, as it is possible that env glycoproteins have toxic effects for the producer cells in the long term which may lead to a decrease of expression.

Such an enhancement of viral production observed with the packaging systems described herein might increase the emergence of unwanted retroviruses having recombined between the genomes of both the retroviral vector and either of the

two packaging-deficient constructs. However, the constructs have been designed in such a way that it reduces the probability of emergence of recombinant viruses compared to the parental constructs. To check their safety, attempts have been made to detect the presence of replication-competent retroviruses by a mobilisation assay of a lacZ provirus. No RC viruses have been found in all retroviral vector preparations tested so far.

The following Examples illustrate the invention.

Example 1

Preparation of Cell lines and viruses.

The following cell lines were used:
A204 (ATCC HTB 82), HeLa (ATCC CCL2), HT1080 (ATCC CCL121), MRC5 (ATCC CCL171), T24 (ATCC HTB 4), VERO (ATCC CCL81) and D17 (ATCC CCL183) were purchased from ATCC.

HOS, TE671 and Mv-1-Lu cells and their clones harboring MFGnlslacZ retroviral vector as described by Takeuchi et al., J Virol (1994), 68, 8001-8007.

The above cell lines were grown in DMEM (Gibco-BRL, U.K.) supplemented with 10% fetal calf serum.

EB8 (Battini et al., J. Virol (1992) 66: 1468-1475);
psiCRE, psiCRELLZ and psiCRIP (Danos et al., Proc. Natl. Acad. Sci USA (1988) 85: 6460-6464);
Cells GP+EAM12 (Markowitz et al., Virology (1988), 167, 400-406); and
NIH-3T3 murine fibroblasts.

These cell lines were grown in DMEM (GIBCO-BRL, U.K.) supplemented with 10% new-born calf serum.

Mv-1-Lu, TE671 and HT1080 cells were transfected using calcium-phosphate precipitation method (Sambrook et., "Molecular Cloning" 1989, Cold Spring Harbour Laboratory Press: New York) as described elsewhere (Battini et al., supra). CeB-transfected Mv-1-Lu, TE671 and HT1080 cells were selected with 3, 6-8 and 4 μg/ml of blasticidin S (ICN, UK), respectively, and blasticidin-resistant colonies were isolated 2-3 weeks later. Cells transfected with the various env-expression plasmids were selected with phleomycin (CAYLA, France): 50 μg/ml (for FBASALF-transfected cells) or 10 μg/ml (for FBASAF-, FbdelPASAF-, FbdelPMOSAF, FBdelPIOAISAF or FBdelPRDSAF-transfected cells). Phleomycin-resistant colonies were isolated 2-3 weeks later.

Production of lacZ pseudotypes using replication competent viruses, amphotropic murine leukemia virus (MLV-A) 1504 strain and cat endogenous virus RD114, was carried out as described previously (Takeuchi et al., J Virol (1994), **68**, 8001-8007).

Example 2

Preparation of Plasmids.

The env gene of pCRIP (Danos et al., supra) was excised by HpaI/ClaI digestion. A 500 bp PCR-generated DNA fragment was obtained using pSV2-bsr (Izumi et al., Experimental Cell Research (1991), **197**, 299-233) as template and a pair of oligonucleotides: (5'>CGGAATTCGGATCCGAGCTCGGCCCAGCCGGCCACCATGAAAACATTTAACATTTCTC) (SEQ ID NO 2) at 5' end and (5'>GATCCATCGATAAGCTTGGTGGTAAAACTTTT) (SEQ ID No 3) at 3' end, with SfiI and ClaI sites, respectively. This fragment was inserted in HpaI/ClaI sites of pCRIP by co-ligation with a 85 bp HpaI/SfiI DNA fragment isolated from pOXEnv (Russell et al., Nucleic Acids Research (1993), **21**, 1081-1085) which

provides the end of the Moloney murine leukemia virus (MoMLV) pol gene. The resulting plasmid named CeB (Fig. 1) could express the MoMLV gag-pol gene as well as the bsr selectable marker conferring resistance to blasticidin S, both driven by the MoMLV 5'LTR promoter.

A series of env-expression plasmids was generated using the 4070A MLV (amphotropic) env gene (Ott et al., J Virol (1990), 64, 757-766) and the FB29 Friend MLV promoter (Perryman et al., Nucleic Acid Res (1991), 19, 6950). In FBASALF (Fig. 1) a BglII/ClaI fragment containing the env gene was cloned in BamHI/ClaI sites of plasmid FB3LPh which also contained the C57 Friend MLV LTR driving the expression of the phleo selection marker. A 136 bp env fragment was generated by PCR using plasmid FB3 (Heard et al., J Virol (1991), 65, 4026-4032) as template and a pair of oligonucleotides: (5'>GCTCTTCGGACCCTGCATTC) (SEQ ID NO 4) at 5' end (before ClaI site) and (5'>TAGCATGGCGCCCTATGGCTCGTACTCTATAGGC)(SEQ ID NO 5)at 3' end, providing a KasI restriction site immediately after the env stop codon. This PCR fragment was digested using ClaI and KasI. A DNA fragment containing the FB29 LTR and the MLV-A env gene was obtained by NdeI/ClaI digestion of FBASALF. The fragments were co-ligated in NdeI/KasI digested pUT626 (kindly provided by Daniel Drocourt, CAYLA labs, France). In the resulting plasmid, named FBASAF (Fig. 1), the phleo selectable marker was expressed from the same mRNA as the env gene. A BglII restriction site was created after the MscI site at position 214 in the FB29 leader by using a commercial linker (Biolabs, France). A NdeI/BglII fragment containing the FB29 LTR was co-inserted with the BglII/ClaI env fragment in NdeI/ClaI-digested FBASAF plasmid DNA, resulting in plasmid FBdelPASAF (Fig. 1). Compared to FBASAF, FBdelPASAF has a 100bp larger deletion in the leader region.

Example 3

Cloning and Sequencing of the RD114 env gene

The RD114 env gene was first sub-cloned in plasmid Bluescript KS+ (Stratagene) as a 3 Kb HindIII insert isolated from SC3C, an RD114 infectious DNA clone (Reeves et al., J. Virol (1984), 52, 164-171). A 2.7 kb ScaI-Hind III fragment of this subclone containing the RD114 env gene was sequenced (Figure 4 (SEQ ID NO 1)- EMBL accession number; X87829). The 5′ non-coding sequence upstream of an NdeI site was deleted by an EcoRI/NdeI digestion followed by filling-in with Klenow enzyme and self-ligation. From this plasmid, two DNA fragments were obtained: a BamHI/NcoI 2.5 Kb fragment and a 63 bp PCR-generated DNA fragment using (5′>CGCCTCATGGCCTTCATTAA) (SEQ OD NO 6) at 5′ end (before NotI site) and (5′>TAGCATGGCGCCTCAATCCTGAGCTTCTTCC) (SEQ ID NO 7) at 3′ end, providing a KasI restriction site just after RD114 env gene stop codon. The PCR fragment was digested with NcoI and KasI. Both fragments were co-inserted between BglII and KasI sites of FBdelPASAF and the resulting plasmid was named FBdelPRDSAF (Fig. 1).

Plasmid pCRIPAMgag- (Danos, O. et al., Proc Natl Acad Sci USA (1988) 85:6460-6464) was used for transfection.

Example 4

Infection assays.

Target cells were seeded in 24-multiwell plates ($4x10^4$ cells per well) and were incubated overnight. Infections were then carried out at 37°C by plating 1 ml dilutions of viral supernatants in the presence of 4 μg/ml polybrene (Sigma) on target cells. 3h later virus-containing medium was replaced by fresh medium and infected cells were incubated for two days before X-gal staining, performed as previously described (Tailor et al., J Virol (1993), 67, 6737-6741,

Takeuchi et al., J Virol (1994), 68, 8001-8007). Viral titers were determined by counting lacZ-positive colonies as previously described (Cosset et al., J. Virol. (1990) 64: 1070-1078). Stability of lacZ pseudotypes in fresh human serum was examined by titrating surviving virus after incubation in 1:1 mixture of virus harvest in serum-free medium and fresh human serum for 1 h at 37°C as described before (Takeuchi et al. supra).

Example 5

Reverse transcriptase (RT) assay.

RT assays were performed either as described previously (Takeuchi et al. supra) or using an RT assay kit (Boehringer Mannheim, U.K.) following the manufacturer's instruction but using $MnCl_2$ (2 mM) instead of $MgCl_2$.

Example 6

Screening producer cell lines.

Viral particles generated with RD114 envelopes have been found to be more stable in human serum than virions with MLV-A envelopes and that the producer cell line also controls sensitivity (Takeuchi et al. supra). A panel of cell lines was screened for their ability to produce high titer viruses and for the sensitivity of these virions to human serum. To do this, cells were infected at high multiplicity with lacZ pseudotypes of either MLV-A or RD114 and cells producing helper-positive lacZ pseudotypes were established. Human HT1080 and TE671 and mink Mv-1-Lu cells were found to release high titer lacZ(RD114) and lacZ(MLV-A) viruses. LacZ(MLV-A) pseudotypes produced by HT1080 cells were more resistant to human serum than those produced by other cells. The titer of these viruses was only four-fold less following a 1 hr incubation with human serum than a

control incubation (Table 1). LacZ(RD114) pseudotypes produced by human cells or mink Mv-1-Lu cells were in general stable in human serum (Table 1). These results suggested that HT1080, TE671 and Mv-1-Lu cells provided the best combination of high lacZ titers and resistance to human serum and they were therefore used for the generation of retroviral packaging cells.

Table 1. Titer and stability of lacZ pseudotypes.

Producer cell	LacZ(MLV-A)		LacZ(RD114)	
	Titer[a]	Stability[b]	Titer[a]	Stability[b]
A204	650	<3	1,200	105
HeLa	9	nd	2,000	115
HOS	4,500	6	23,000	86
HT1080	2,000,000	26	400,000	129
MRC-5	450	10	1,000	nd
T24	350	nd	1,200	nd
TE671	15,000	2	90,000	38
VERO	260	nd	90	nd
D17	900	<1	200,000	1
Mv-1-Lu	80,000	1	200,000	120

a: titration on TE671 cells as lacZ i.u./ml
b: % of infectivity of human serum-treated viruses compared to fetal calf serum-treated viruses

Example 7

Construction of an improved gag-pol expression vector.
A MoMLV gag-pol expression plasmid, CeB (Fig. 1), was

derived from pCRIP (Danos et al., Proc. Natl. Acad Aci USA (1988) 85: 6460-6464). Approximately 2 Kb of env sequence were removed from pCRIP and the bsr selectable marker, conferring resistance to blasticidin S (Izumi et al., Experimental Cell Research (1991) 197:229-233), was inserted 74 nts downstream of the gag-pol gene. This 74 nts interval had no ATG triplets and was thought to provide an optimal distance between the stop codon of the pol reading frame and the start codon of the bsr gene to allow re-initiation of translation (Kozak Mol Cell Biol., 1987, 7: 3438-3445). There was no "Kozak" consensus sequence (Kozak Cell, (1986) 44: 283-292) at the 5' end of the marker gene. Therefore, bsr could only be expressed by re-initiation of translation after the upstream gag-pol gene had been expressed. Consequently, after transfection of CeB in Mv-1-Lu/MFGnlsLacZ (ML), TE671/MFGnlsLacZ (TEL) or HT1080 cells, blasticidin S-resistant bulk populations and most cell clones expressed high levels of gag-pol proteins assessed by the reverse-transcriptase (RT) activity found in cell supernatants (Table 2). Considerably higher RT activities were found in bulk populations of CeB-transfected ML cells compared to bulk population of ML cells stably transfected with the parental pCRIP construct. Similarly the RT activities of two packaging cell lines generated using pCRIPenv- construct, psiCRE cells (Danos et al., supra) and EB8 cells (Battini supra.) were less than that of CeB transfected clones (Table 2). Finally, RT activitiy in CeB transfected cell supernatants was higher than that of cells chronically infected by replication-competent MLV-A (Table 2).

Table 2. Secreted reverse transcriptase expression

Cell[a]	RT activity[b]	LacZ Titer[c]

ML/MLV-A	1	$8x10^4$
MLSvB	0.1	<1
MLCRIP (bulk)	0.15	nd
MLCeB (bulk)	1.7	nd
MLCeB1	4.2	$1x10^6$
MLCeB4	1.6	$1x10^6$
TEL/MLV-A	3.6	$2x10^6$
TELCeB6	5.2	$4x10^7$
HT1080/MLV-A	1.1	$1x10^6$
HTCeB6	1.9	$1x10^6$
HTCeB18	2.7	$2x10^6$
HTCeB22 (FLY)	6.9	$5x10^6$
HTCeB48	5.5	$3x10^6$
EB8	0.22	$1x10^4$
psiCRE-LLZ	1.2	$1x10^{5}$[d]

a: ML, Mv-1-Lu cells harboring a MFGnlslacZ provirus; TEL, TE671 cells harboring a MFGnlslacZ provirus; /MLV-A, cells chronically infected with MLV-A 1504 strain; MLSvB, ML cells transfected with a plasmid pSV2bsr alone; MLCRIP, ML cells co-transfected with pCRIP and pSV2bsr.
b: Average of arbitrary units relative to ML/MLV-A RT activity of at least two independent experiments was shown. The standard errors did not exceed 20 % of the values.
c: titration on TE671 cells as lacZ i.u./ml. After polyclonal transfection of a plasmid which expresses MLV-A env in MLCeB clones, TELCeB clones, HTCeB clones and EB8 cells; nd, not done.
d: titration on NIH3T3 cells

To rescue infectious lacZ viruses, MLCeB and TELCeB clones were transfected with FBASALF DNA, a plasmid designed to express the MLV-A env gene (Fig. 1). Bulk populations of stable FBASALF transfectants were isolated and supernatants were titrated using TE671 cells as targets. Titers of lacZ viruses were higher than either MLV-A infected ML or TEL cells, or FBASALF-transfected EB8 cells (Table 2). These data suggested that CeB was an extremely efficient MLV gag-pol expression vector in mink Mv-1-Lu and TE671 cells. CeB

was therefore used to derive packaging cells by transfection of HT1080 cells. 41/49 blasticidin S-resistant colonies had detectable levels of RT; 9 had RT activity higher than that of control MLV-A-infected HT1080 cells (data not shown). Expression of gag precursor was confirmed in cell lysates and supernatants of these 9 HTCeB clones by immunoblotting using antibodies against p30-CA (data not shown). The 4 clones with the highest expression of gag proteins (clones 6,18,22 and 48) were infected at high-multiplicity with helper free, lacZ pseudotypes bearing MLV-A envelopes (MFGnlslacZ(A)) produced by TELCeB6/FBASALF (Table 3) and then transfected with FBASALF. Supernatants of bulk, phleomycin-resistant transfectants were assessed for RT activity and lacZ titer (Table 2). Clone HTCeB22, named FLY, was found to be the best gag-pol producer clone and was used to introduce env expression vectors for the generation of packaging cell lines.

Table 3. Titer following env construct transfection

Producer cell	Env source	Titer[a]
psiCRIP lacZ 5	pCRIPAMgag-	$6x10^{4b}$
GP+EAM12 lacZ 25	envAM	$3x10^{5b}$
TELCeB6	FBASALF[c]	$5x10^7$
	FBASAF[c]	$2x10^7$
	FbdelPASAF[c]	$2x10^7$
TELCeB6	FBdelPASAF 1	$3x10^7$
	FbdelPASAF 4	$2x10^7$
	FbdelPASAF 6	$1x10^7$
	FbdelPASAF 7	$5x10^7$
	FbdelPASAF 8	$1x10^7$
	FbdelPRDSAF 2	$1x10^6$
	FbdelPRDSAF 4	$3x10^5$
	FbdelPRDSAF 7	$1x10^7$
	FbdelPRDSAF 8	$2x10^6$
FLY[d]	FBdelPASAF 1	$1x10^1$
	FbdelPASAF 4	$1.5x10^6$
	FbdelPASAF 5	$1x10^6$
	FbdelPASAF 7	$1x10^6$
	FbdelPASAF 13	$7x10^6$
	FbdelPASAF 14	$4x10^6$
	FbdelPASAF 15	$1x10^6$
	FbdelPASAF 16	$5x10^6$
	FbdelPASAF 17	$6x10^6$
FLYA4 lacZ 3	FBdelPASAF 4	$2x10^{7b}$
FLY[d]	FBdelPRDSAF 1	$2.5x10^6$
	FbdelPRDSAF 2	$1x10^7$
	FbdelPRDSAF 6	$5x10^6$
	FbdelPRDSAF 10	$2x10^6$
	FbdelPRDSAF 11	$3x10^6$
	FbdelPRDSAF 13	$1x10^6$
	FbdelPRDSAF 17	$5x10^6$
	FbdelPRDSAF 18	$3x10^7$
	FbdelPRDSAF 19	$6x10^6$

Average titers of at least three independent experiments were shown. The standard errors did not exceed 30 % of the titer values.
a: titrated on TE671 cells as lacZ i.u./ml
b: results of best MFGnlslacZ producer clones.

c: bulk populations of env-transfectants in TELCeB6 cells.
d: titration after bulk infection with helper-free MFGnlslacZ.

Example 8

Construction of env expression vectors.
A series of MLV-A env expression plasmids were then generated (Fig. 1). In FBASALF, the env gene was inserted between two Friend-MLV LTRs, its expression driven by the FB29 MLV LTR (Perryman et al., supra). Most of the packaging signal located in the leader region was deleted. This plasmid also expressed the phleo selectable marker (Gatignol et al., supra) driven by the 3′ LTR. FBASAF and FBdelPASAF were then designed following the same strategy used for CeB. These two vectors differed only by the extent of deletion of the packaging signal, FBdelPASAF having virtually no leader sequence. Compared to pCRIPAMgag- and pCRIPgag-2 env plasmids expressed in psiCRIP or psiCRE packaging cells (Danos et al., supra) about 5 Kb of gag-pol sequences was removed. In addition the 258 bp retroviral sequence containing the end of env gene and the begining of U3 found in pCRIPAMgag- and pCRIPgag-2 was also removed. For both FBASAF and FBdelPASAF plasmids, the phleo selectable marker was inserted downstream of the env gene by positioning a 76 nts linker with no ATG codons between the two open-reading frames. Phleo could therefore only be expressed by re-initiation of translation by the same ribosomal unit that had expressed the upstream env open reading frame. FBdelPASAF was also used to generate FBdelPRDSAF, an RD114 envelope expression plasmid (Fig. 1).

After transfection of the env plasmids into TELCeB6 cells (Table 2), bulk populations of phleomycin-resistant colonies were isolated and their production of lacZ virus measured

(Table 3). FBASALF gave a titer of $5x10^7$ lacZ-i.u./ml, whilst titers with either FBASAF or FBdelPASAF were $2x10^7$ lacZ-i.u./ml (Table 3). Titers of $5x10^7$ or 10^7 lacZ-i.u./ml could be obtained with some FBdelPASAF cell clones or FBdelPRDSAF clones, respectively.

As FBdelPASAF has minimal virus-derived sequences and was shown to be the safest construct (see below and Table 4), it and FBdelPRDSAF were used to generate packaging lines from FLY cells (clone HTCeB22, Table 2). Envelope expression of these clones was assayed by interference to challenge with MFGnlslacZ(A) or MFGnlslacZ(RD) pseudotypes produced by TELCeB6/FBdelPASAF-7 or TELCeB6/FBdelPRDSAF-7, respectively (Table 3). The cell lines showing most interference were cross-infected at high multiplicity with these pseudotypes to provide MFGnlslacZ proviruses, and supernatants were then titrated on TE671 cells (Table 3). FLY-FBdelPASAF-13 (FLYA13 packaging line) and FLY-FBdelPRDSAF-18 (FLYRD18 packaging line) gave the highest productions of lacZ viruses, around 10^7 lacZ-i.u./ml. The best MFGnlslacZ producer clones derived from either psiCRIP cells (Danos et al., supra) or GP+EAM12 cells (Markowitz et al., supra) gave approximately 50 fold lower titers (Table 3). The lacZ titers of the FLY-derived lines shown in Table 3 are lower than the best TELCeB6-derived lines after transfection of either FBdelPASAF or FBdelPRDSAF (Table 3). However it should be noted that the lacZ provirus expressed in TELCeB6 cells was obtained after clonal selection but was introduced polyclonally in FLY-derived env-transfected cell clones. When FLY-FBdelPASAF-4 cells (FLYA4 packaging line), infected with helper-free MFGnlslacZ(RD), were cloned by limiting dilution the best clones (eg. FLYA4lacZ3) were found to produce 20 times more infectious viruses than the bulk population, reaching the range of titers obtained with the best TELCeB6-FBdelPASAF clones (Table 3).

Example 9

Assays for transfer of gag-pol or env functions.
To assay for replication-competent viruses, supernatants were used to infect TEL cells (a clone of TE671 cells harboring an MFGnlslacZ provirus). Infected cells were passaged for 6 days or longer and their supernatants were used for infection of fresh TE671 cells. No transmission of lacZ viruses could be detected (Table 4), demonstrating that the supernatants of pCRIPAMgag--, FBASALF-, FBASAF-, or FBdelPASAF-transfected TELCeB6 cells were helper-free. Similar absence of replication competent recombinant retroviruses was demonstrated using supernatant from a clone of psiCRIP-MFGnlslacZ cells or from two clones of FLYA-MFGnlslacZ cells (Table 4).

There have been reports that helper-free retroviral vector stocks may nevertheless contain recombinant retroviruses (replication incompetent) carrying either gag-pol or env genes (Bestwick et al., Proc Natl Acad Sci USA (1988), **85**, 5404-5408, Cosset et al., Virology (1993), **193**, 385-395, Girod et al., Virology (1995), **in press**). To assay for such recombinant retroviruses, mobilisation of an MFGnlslacZ provirus from two indicator cell lines which could cross-complement potential recombinant viruses carrying either gag-pol or env functional genes was attempted. The TELCeB6 line (Table 2) expressing gag-pol proteins was used as indicator cell line to test for the presence of env recombinant (ER) viruses. The TELMOSAF indicator line expressing MoMLV env glycoproteins (obtained by transfection of FBMOSAF, a plasmid expressing the MoMLV env gene using FBASAF backbone, in TEL cells) was used to detect the presence of gag-pol recombinant retroviruses (GPR viruses). After passaging 4-8 days, the supernatants of the infected indicator cells were used to infect either human TE671 cells

or murine NIH3T3 cells.

TELCeB6 cells transfected with various env-expressing constructs, pCRIPAMgag-, FBASAF and FBdelPASAF were compared. Although the supernatants of TELCeB6-FBdelPASAF cells were devoid of replication-competent retroviruses, they were found sporadically to transfer gag-pol genomes (Table 4). No GPR viruses could be detected when less than $2x10^5$ virions were used to infect the indicator cells. Similarly TELCeB6 indicator cells infected with various helper-free viruses were shown sporadically to release lacZ virions (Table 4). The number depended both on the env-expression vector used and on the virus input quantity. Compared to lacZ viruses generated using pCRIPAMgag-plasmid, the frequency of detection of the env-recombinant viruses was lower for supernatants generated by using FBASAF and FBdelPASAF constructs (Table 4). For FBdelPASAF construct when less than $5x10^5$ MFGnlslacZ(A) helper-free virions were used to infect the indicator cells, no ER retroviruses could be detected. From these experiments, it could be estimated that a supernatant, produced from TELCeB6-FBdelPASAF cells, containing $1x10^7$ infectious units of MFGnlslacZ retroviral vector contained no replication-competent virus, and about 100 gag-pol and 100 env recombinant retroviruses.

Table 4. Transfer of packaging function

Producer cell	Indicator cell	Input virus[a] (lacZ-i.u.)	Detection[b] ++	+	-
		Replication competent virus			
psiCRIP lacZ 5	TEL	$2x10^4$	0/4	0/4	4/4
TELCeB6-pCRIPAMgag-	TEL	$5x10^6$	0/4	0/4	4/4
TELCeB6-FBASAF	TEL	$5x10^6$	0/4	0/4	4/4
TELCeB6-FBdelPASAF	TEL	$5x10^6$	0/4	0/4	4/4
FLYA4 lacZ 3	TEL	$1x10^7$	0/4	0/4	4/4
FLYA4 lacZ 7	TEL	$1x10^7$	0/4	0/4	4/4
		Gag-pol recombinant			
TELCeB6-FBdelPASAF 7	TELMOSAF	$2x10^7$	0/4	1/4	3/4
TELCeB6-FBdelPASAF 7	TELMOSAF	$2x10^6$	0/4	2/4	2/4
TELCeB6-FBdelPASAF 7	TELMOSAF	$2x10^5$	0/4	2/4	2/4
TELCeB6-FBdelPASAF 7	TELMOSAF	$2x10^4$	0/4	0/4	4/4
		Env recombinent			
TELCeB6-pCRIPAMgag-	TELCeB6	$5x10^6$	2/4	1/4	1/4
TELCeB6-pCRIPAMgag-	TELCeB6	$5x10^5$	1/4	1/4	2/4
TELCeB6-pCRIPAMgag-	TELCeB6	$5x10^4$	0/4	2/4	2/4
TELCeB6-FBASAF	TELCeB6	$5x10^6$	0/4	2/4	2/4
TELCeB6-FBASAF	TELCeB6	$5x10^5$	0/4	1/4	3/4
TELCeB6-FBASAF	TELCeB6	$5x10^4$	0/4	1/4	3/4
TELCeB6-FBdelPASAF	TELCeB6	$5x10^6$	0/4	1/4	3/4
TELCeB6-FBdelPASAF	TELCeB6	$5x10^5$	1/4	3/4	0/4
TELCeB6-FBdelPASAF	TELCeB6	$5x10^4$	0/4	0/4	4/4

a: number of lacZ i.u. used to infect indicator cells
b: number of incidence out of four experiments. The ranges of lacZ titers rescued from infected indicator cells are shown for each virus input: >100 lacZ i.u./ml (++), 1-100 lacZ i.u./ml (+) and <1 lacZ i.u./ml (-).

Titers were determined on TE671 cells for replication competent virus and env recombinant and NIH3T3 cells for

gag-pol recombinant.

Example 10

In order to confirm resistance to complement and absence of replication competent virus in our best packaging lines, MFGnlslacZ(A) and (RD) harvested from FLYA13 and FLYRD18, respectively, after polyclonal transduction of MFGnlslacZ (Table 3 above) were tested for stability in fresh human serum and generation of replication competent virus. Titers of MFGnlslacZ(RD) from FLYRD18 after 1 hr incubation with 3 independent samples of fresh human serum were 80 to 120 % of control incubations, while titers of MFGnlslacZ(A) from FLYA13 were 50 to 90 % of controls (data not shown). No replication competent virus was detected in the same assay described above (Table 4) when 1 x 10^7 i.u. each of MFGnlslacZ(A) and (RD) were tested.

EXAMPLE 11.

Generation of plasmids.

CeB plasmid (Fig. 5) expressing MoMLV gag-pol gene, was further modified to remove the splice donor site located in the leader region. A 272 bp fragment was PCR-generated by using OUSD- (5'-TCTCGCTTCTGTTCGCGCGC) and OLSD- (5'-TCGATCAAGCTTGCGGCCGCGGTGGTGGGTCGGTGGTCC) as primers and further digested with BssHII and HindIII. A 1008 bp HindIII-XhoI fragment isolated from CeB (encompassing a part of leader sequence and beginning MoMLV gag) and the PCR fragment were co-inserted into pCeB from which the 1275 bp BssHII-XhoI fragment (encompassing R-U5-leader-gag) had been removed. The resulting plasmid, named pCeB DS- (Fig. 5), beared the deletion of splice donor (SD) site and a NotI restriction site created just downstream to the lost SD site.

A series of gag-pol expression plasmids in which the MoMLV LTR promoter was replaced by the human cytomegalovirus immediate early promoter (hCMV promoter) was derived from both CeB DS- and hCMV-G (Yee et al., 1994 PNAS, 91: 9564-9568), a plasmid used as a source for the hCMV promoter. A NotI-filled/EcoRI 7260 bp fragment was isolated from CeB DS- and cloned into hCMV-G which had been opened with SalI (further rendered blunt-ended) and EcoRI to remove the VSV-G gene. The resulting plasmid was cutted with ClaI and EcoRI to remove a 1155 bp fragment encompassing sequence derived from 3'-LTR and SV40 polyA sequence and self-ligated after filling both protruding DNA ends. The resulting plasmid, named phCMV-intron (Fig. 5), had gag-pol and bsr ORFs inserted between the CMV promoter and rabbit beta-globin polyA post-transcriptional regulatory sequences.

An intermediate plasmid was generated by sub-cloning a 7260 bp EcoRI fragment (isolated from CeB DS-) into hCMVG opened with EcoRI. A 1155 bp fragment (encompassing sequence derived from 3'-LTR and SV40 polyA sequence) was removed from this intermediate plasmid which was then re-circularized by self ligation after filling both ends. The resulting plasmid, named phCMV+intron 2P (Fig. 5), was digested with NotI and the vector was treated with klenow enzyme. A 1440 bp fragment (encompassing hCMV promoter and rabbit beta-1 globin intron B (Rohrbaugh et al., 1985 Mol. Cell Biol, 5: 147-160)) was isolated from phCMV+intron 2P by NotI/EcoRI digestion. This fragment was further treated with klenow enzyme and ligated back into the vector. The resulting plasmid, named hCMV+intron (Fig. 5), could express gag-pol and bsr genes driven by the hCMV promoter and beared an intron sequence derived from rabbit beta-1 globin intron B having both SD and SA (splice acceptable) sites.

A 2450 bp fragment was removed from phCMV+intron 2P by

NotI/XhoI digestion. The resulting vector fragment was then used to co-ligate a 1330 bp fragment (containing hCMV promoter + 5′ end of rabbit beta-1 globin intron B (with SD site)) isolated from phCMVG by ApaI-filled/NotI digestion and a 1 kb fragment isolated from phCMV+intron 2P by NotI-filled/XhoI digestion. Compared to phCMV+intron 2P, the resulting plasmid, named hCMV+SD intron (Fig. 5), had the deletion of the 3′ end of the rabbit beta-1 globin intron B and thus no SA site in the leader region.

Construct phCMV+leader (Fig. 5) has been described elsewhere (Savard et al., unpublished). This plasmid, in which gag-pol and bsr genes were driven by the hCMV promoter, had the MoMLV SD site in the leader region.

Gag-pol expression.

The different constructs, including the parental CeB plasmid, were analysed comparatively in a complementation assay after transfection in TEL-FBdelPASAF cells expressing 4070A-MLV (amphotropic) envelope and harboring a MFGnlslacZ provirus. The transient production of lacZ retroviruses as well as the stable production of lacZ retroviral vectors after selection with blasticidin S were determined (Table 5). All the constructs were able to rescue infectious lacZ retroviruses indicating the expression of gag-pol proteins after transient transfection. Most likely due to the efficient hCMV and rabbit beta-1 globin intron B (post)-transcriptional regulatory sequences, hCMV+intron was particularly potent in transient retroviral vector production. However, 10 times less blasticidin-resistant colonies were obtained with hCMV+intron comparatively to CeB, and stable lacZ virus production from hCMV+intron was about 5-10 times lower than that of CeB. Clonal examination of lacZ retrovirus production from blasticidin-resistant colonies indicated that 80-90% of colonies could express

high levels of gag-pol proteins for both hCMV+intron and CeB plasmids. In contrast, despite variation in their ability to form blasticidin-resistant colonies after transfection and despite their ability to express gag-pol proteins from transient transfectants, all other constructs had a weak capacity for rescuing lacZ retroviral vectors from stable transfectants (Table 5).

Table 5. Comparative study of gag-pol-bsr plasmids.

gag-pol-bsr plasmid	Transient (lacZ i.u./ml)	no clones bsr^+	Stable (lacZ i.u./ml	% gag-pol /bsr
Ceb	300/ml	50	10^7	90%
Ceb DS-	144/ml	5	10^5	50%
hCMV+intron 2P	ND	20	10^6	50%
hCMV-intron	812/ml	0	-	-
hCMV+SD intron	150/ml	1000	10^2	nd
hCMV+leader	328/ml	1000	10^2-10^3	nd
hCMV+intron	12000/ml	5	10^6-10^7	80%

Northern blot analyses were performed on stable transfectants (blasticidin-resistant) obtained with some of the gag-pol-bsr plasmids. As expected, the results (not shown) displayed a correlation between expression of gag-pol mRNAs and gag-pol protein expression detected by rescue analysis (Table 5). CeB construct was found to produce 2-3 fold more gag-pol mRNAs compared to hCMV+intron. Interestingly, an unexpected 2.45 kb RNA band was found for hCMV+intron construct at a ratio of 2:1 compared to the abundancy of the gag-pol mRNA band (at 5.95 kb). Further

investigations by using other probes revealed that a cryptic splice donnor (SD) site located in the gag gene (right in the middle of the CA coding region at position 1596-1597 -numbering according to Shinnick et al., 1981 Nature (London) 293: 543-548) was activated in this latter construct. The 2.45 RNA species, lacking the 3' half of the gag gene and most of the pol gene, is unlikely to give rise to any useful translational product. It is therefore interesting to notice that hCMV+intron construct was able to give rise to slightly more transcripts (gag-pol 5.95 mRNA + 2.45 alternative RNA band) compared to gag-pol mRNA expressed from CeB construct. Therefore we decided to inactivate the cryptic SD site in the hCMV+intron construct in order to increase the ratio of gag-pol mRNAs.

Assays for transfer of gag-pol functions.

Although the supernatants of pacakaging cell lines generated with CeB gag-pol expression contruct were devoid of replication-competent retroviruses, they were found sporadically to transfer gag-pol genomes (example 9, Table 4) (Cosset et al., 1995 J. Virol 69: 7430-7436). Because gag-pol-bsr constructs generated here by using the hCMV promoter had much less retroviral sequences homologous to the retroviral vector than the parental CeB construct (Fig. 5), they are less likely to give rise to gag-pol recombinant (GPR) viruses. Therefore, the most efficient gag-pol-bsr plasmids, hCMV+intron and CeB, were further analysed for emergence of GPR viruses. To assay for such recombinant retroviruses, we attempted to mobilise an lacZ provirus from an indicator cell lines which could cross-complement potential recombinant viruses carrying gag-pol functional genes. Results displayed in Table 6 showed that consistently with data reported previously (example 9, Table 4)(Cosset et al., 1995 Supra), lacZ retrovirus vectors generated by using CeB gag-pol construct were contaminated with GPR viruses. In

contrast lacZ retrovirus vectors generated by using hCMV+intron construct were completely devoid of such GPR viruses, suggesting that this construct was improved compared to CeB with respects with emergence of recombinant viruses.

Table 6. Comparative study of gag-pol-bsr plasmids.

plasmid	input virus (lacZ i.u.)[a]	no of experiments giving titres of[b]		
CeB	$5x10^6$	5	3	0
	$5x10^5$	2	4	2
	$5x10^4$	0	1	7
hCMV+intron	$5x10^6$	0	0	8
	$5x10^5$	0	0	8
	$5x10^4$	0	0	8

4x10E4 cells of TEL/MOSAF in 24 wells were challenged with lacZ(A) of i.u. indicated in the table (a), and incubated at 37°C for 3 days. Cells were trypsinized and transferred into small flasks. Cell sup was harvested on day 5 after lacZ(A) challenge and plated on either TE571 (not shown) and 3T3 cells (b). No lacZ was mobilized into TE671 at all. LacZ(A) from CMV-int 10 again did not rescue lacZ from TEL/MOSAF.

Example 12

Generic primers to detect D-type (Medstrand and Blomberg J.Virol. (1993) 67:6778-6787) , C-type (Shih et al., J Virol. (1989) 63:64-75), human endogenous virus RTVL-H (Wilkinson et al., J.Virol. (1993) 67:2981-2989), by RT-PCR were employed (Patience et al.,supra). Primers to detect mouse endogenous VL30 element (Adams et al Mol.Cel.Biol. (1988) 8:2989-2998), and MFGnlslacZ RNA were designed and synthesized (TABLE X). Overnight supernatants (in 4ml of culture medium) from 106 cells of GP+EAM12lacZ25, FLYA4lacZ3

and TELCeB6FBASALF cells (Table 3) were harvested and centrifuged in sucrose gradient as described previously (Patience et al., J.Virol., 70:2654-2657). Fractions containing retrovirus particles were collected, and RNA extracted. One twentieth of the RNA preparation or dilution's thereof were applied to RT-PCR as described previously (Table X). A 1/200 of RNA harvested from GP+EAM12lacZ25 cells was positive for VL30 RNA. MFGnlslacZ RNA was found from 1/20 of RNA from GP+EAM12lacZ and TELCeB6FBASALF cells and 1/200 of RNA from FLYA4lacZ3 cells. The primer combinations for RTVL-H, C- and D-type RNA did not give detectable PCR product.

Table 7. RT-PCR detection of endogenous retrovirus RNA associated with virus particles.

RNA	primer (5'-3') forward(F)/reverse(R)	rt-pcr of virion associated RNA from[a]		
		GP+EAM12 lacZ25	FLYA4 lacZ3	TELCeB6F BASALF
MFGnls lacZ	F) CTCTGGCTCACAGTACGACGTAG R) CCATCAATCCGGTAGGTTTTCCG	+	++	+
C-type	F) CARRGKTTCAARAACWSYCCCAC R) AGYARVGTAGCNGGGTTHAGG	-	-	-
D-type	F) TCCCCTTGGAATACTCCTGTTTTYGT R) CATTCCTTGTGGTAAAACTTTCCAYTG	-	-	-
RTVL-H	F) CCTCACCCTGATCACRYTTG R) GAATTATGTCTGACAGAAGGG	NT	-	-
VL30	F) GTTGACATCTGCAGAGAAAGACC R) TCTGAGGTCTGTACACACAATGG	++	NT	NT

a:-,not detected; + detected in 1/20 RNA preparation; ++ detected in 1/200 RNA preparation; NT, not tested because the cells do not possess the corresponding genes.

EXAMPLE 13.

Generation of gag-pol pre-packaging cells by using TE671 cells.

CeB, a plasmid designed to over-express MoMLV gag and pol proteins was introduced in TE671 human rhabdomyosarcoma cells (ATCC CRL8805). After selection with blasticidin, 50 bsr-positive colonies were isolated and the RT (reverse transcriptase) activity was analysed in their supernatants. 12 TE671-CeB (TECeB) clones with high RT activity were selected for further analysis. The best TECeB clone, clone #15, had a RT activity roughly equivalent to that TELCeB6 cells (Cosset et al., J. Virol. 69:7430-7436 (1995); see also Example 7, Table 6 in this patent application) but displayed 2-3 fold more gag-precursors into cells as demonstrated in immunoblots by using anti-CA antibodies. The biological activity of gag-pol proteins expressed in the six best TECeB clones was further confirmed by their ability to produce infectious retroviruses in a complementation assay. A lacZ provirus was introduced into each of the TECeB clones by polyclonal cross-infection by using lacZ(RD114) helper-free retrovirus vectors. FBMOSALF, a MoMLV env expression plasmid (Cosset et al., J. Virol. 69:6314-6322), was then transfected in each of the TECeB-lacZ lines and in the TELCeB6 cell line for comparison. After selection with phleomycin, the titer of lacZ retrovirus vectors was determined in the supernantant of pools of phleomycin-resistant colonies for each TECEB-lacZ-FBMOSALF lines. A

good correlation was found between gag-pol expression into the TE-CeB clones (as determined by RT-assays and anti-gag immunoblots) and their ability to release infectious lacZ particles. TE-CeB15 cells could release approximately the same number of lacZ particles when compared to TELCeB6 cells although TELCeB6 cells had the advantage of being selected for lacZ expression (Cosset et al., J. Virol. 69:7430-7436 (1995)). TE-CeB15 cells were therefore used to derive retroviral packaging cell lines.

Construction of env-expression plasmids.

A series of plasmid (Fig. 3) was designed to allow expression of different retroviral envelope genes (isolated from MoMLV, GALV -Gibbon Ape Leukemia Virus-, and MLV-10A1). FBdelPMOSAF (Fig. 3, nucleotide sequence in Fig. 10) and FBdelP10A1SAF, expressing ecotropic MoMLV or MLV-10A1 envelopes, were generated by replacing the BglII/ClaI fragment from FBdelPASAF (Cosset et al., J. Virol. 69:7430-7436 (1995); see also Example 7, Fig. 2 and nucleotide sequence in Fig. 9) encompassing most of the env gene and splice acceptor site with that of MoMLV (position 5407 to 7679, Shinnik et al., 1981) or with that of MLV-10A1 (Ott et al., J. Virol. 64:757-766 (1990)).
Nucleotides 7514-7516 of GALV (Delassus et al., Virology 173:205-213 (1989)) were mutated by PCR-mediated mutagenesis to create a ClaI site (AAG to CGA), thereby introducing a conservative modification (a lysine (amino-acid 665 of GALV env precursor) to an arginine). The BamHI/ClaI fragment (nts 4994 (Delassus et al. Virology 173:205-213 (1989)) to 7517) was then sub-cloned into FBdelPASAF in which the BglII/ClaI encompassing most of the env gene and splice acceptor site had been removed. The resulting plasmid, expressing GALV

envelope glycoproteins, was named FBdelPGASAF (Fig. 3, nucleotide sequence in Fig. 11).

CMV10A1 was generated by inserting a Klenow enzyme-filled EagI/SalI fragment from FBdelP10A1SAF (encompassing 10A1 MLV env gene and phleo selectable marker) into hCMV-G digested with BamHI and filled with Klenow enzyme. The resulting plasmid, CMV10A1 (Fig. 3 and nucleotide sequence in Fig. 13) could express 10A1 envelopes under control of the hCMV promoter and the phleo selectable marker by translation re-initiation.

Generation of a multi-tropic set of TE671-based retroviral packaging lines.

FBdelPRDSAF (Fig. 3, nucleotide sequence in Fig. 12), FBdelPASAF, FBdelPGASAF, FBdelPMOSAF and FBdelP10A1SAF were independently introduced into cells of the TE-CeB15 pre-packaging line, expressing MoMLV gag-pol proteins. Transfected cells were phleomycin-selected and 15-20 phleo-resistant colonies were isolated for each env-expression plasmid transfected.

Individual colonies were then analysed for expression of envelope glycoproteins by immunoblots on cell lysates by using antibodies against RD114 SU glycoproteins or against Rausher leukemia virus SU (to screen MoMLV, MLV-4070A and MLV-10A1 env-producer clones) or against GALV. The best env-producer colonies as determined in this assay were further analysed by a complementation assay after introducing a lacZ retroviral vector. LacZ pseudotypes released from the different packaging cell lines were titrated by using NIH 3T3 cells or TE671 cells as target. Titers higher than $1x10^7$ lacZ i.u./ml were obtained for the best clones. Depending on the envelope specificities expressed in these cells, the new

TE671-based retroviral packaging cell lines were named TE-FLYE, TE-FLYA, TE-FLYRD, TE-FLY10A1, and TE-FLYGA and could express the MoMLV, MLV-4070A, RD114, MLV-10A1, and GALV env genes, respectively.

Assays for detecting replication-competent retroviruses (RCRs) were performed in the supernatants of these cells and were negative (less than 1/ml).

TE671 cells are very potent for transient expression resulting in more than 95% of cells expressing transgene three days after plasmid transfection (Hatziioannou and Cosset, unpublished data, (1996)). The ability of retroviral packaging cell lines to transiently produce retroviral vectors is of crucial importance for gene therapy where vectors carrying toxic gene have to be prepared. Transient expression of retroviral vectors was comparatively determined from cells of the TE-FLYA line and from the BING line (Pear et al., Proc Natl Acad Sci U S A 90, 8392-6 (1993)), a retroviral packaging cell line designed to transiently express retroviral vectors. Results (Table 8) showed that TE-FLYA cells were more efficient for transient expression of a lacZ retroviral vector hence resulting in higher titers.

Table 8. Comparative study of transient production of lacZ vectors.

packaging cell line	cell number[a]	% transfected cells[b]	transient titer[c]
BING	281	5.3	$2x10^2$
TE-FLYA	117	35	$1.3x10^3$

Cells were transfected by MFGnislacZ retroviral vectors with calcium phosphate precipitation method and titers of of lacZ vectors (c) released in cell

supernatant were determined as lacZ i.u./ml at day 3 following transfection. The relative number of cells (a) (average per microscope field) and the % of transfected cells (b) determined after X-gal staining are shown.

Retroviral vectors prepared from TE671-based packaging cell lines were analysed for their sensitivity to human-complement mediated inactivation. Experiments were conducted as previously described (Cosset et al., J. Virol. 69:7430-7436 (1995); see also Example 10 in this patent application) by using three human sera of individual donors (Table 9). As expected MLV-A prepared from mouse 3T3 cells were highly sensitive to inactivation after 1 hr incubation witn sera. In contrast, titers of lacZ vectors produced from TE-FLYRD cells were 17 to 55% of control incubations, while titers of lacZ vectors from TE-FLYA cells were 1 to 30% of controls.

Table 9. Human serum sensitivity of viruses produced from TE671-based packaging cell lines.

Virus from:	**hu56[a]**	**hu57[a]**	**BTS[a]**
3T3/A	<0.2, <0.2	<0.2, <0.2	<0.2, <0.2
TE-FLYE	15, 7.8	16, 11	48, 60
TE-FLYA	1, 0.6	2.2, 7.1	28, 19
TE-FLYRD	17, 22	30, 44	54, 63

Three human fresh serum samples were tested in duplicate; hu56 (A+), hu57(AB+), BTS(AB+). (a) % control (average for FCS and opti-MEM treatment) is shown.

CLAIMS:

1. A recombinant expression vector comprising a gene of interest and a selectable marker gene, wherein the selectable marker gene is arranged downstream of the gene of interest and a stop codon associated with the gene of interest is spaced from a start codon of said selectable marker gene at a distance which is sufficient to ensure that said selectable marker protein is expressed from the corresponding mRNA as a result of translation reinitiation.

2. A recombinant expression vector according to claim 1 wherein the vector is a viral vector.

3. A recombinant expression vector according to claim 2 wherein the vector is a retroviral vector.

4. A recombinant expression vector according to any one of claims 1 to 3 wherein the gene of interest is included as part of a viral packaging construct.

5. A recombinant expression vector according to any one of the preceding claims wherein the number of nucleotides in the space between the stop codon of the gene of interest and the start codon of the selectable marker is in the range of from 20 to 200 nucleotides.

6. A recombinant expression vector according to claim 5 wherein the number of nucleotides in the space between the stop codon of the gene of interest and the start codon of the selectable marker is in the range of from 60 to 80 nucleotides.

7. A process for producing a cell line in which a gene of interest is expressed, which process comprises:

transforming host cells with an expression vector

according to any one of the claims 1 to 6; and

selectable those cells where expression of the selection marker gene may be detected.

8. A process according to claim 7 wherein the host cell is a eukaryotic cell.

9. A host cell transformed with a recombinant expression vector according to any one of the claims 1 to 6.

10. A retroviral packaging cell line comprising a host cell transformed with a first and a second recombinant expression vector, said first recombinant expression vector having a packaging-deficient construct comprising a viral gag-pol gene and a first selectable marker gene downstream thereof, and said second recombinant expression vector having a packaging-deficient construct comprising a viral env gene and a second selectable marker gene downstream thereof; wherein the start codon of the first and second selectable markers are spaced from the stop codons of the viral gag-pol gene and the viral env gene respectively by a distance which ensures that said selectable marker protein is expressed from the corresponding mRNA as a result of translation reinitiation.

11. A retroviral packaging cell line according to claim 10 wherein the first selectable marker is a bsr selectable marker and the second selectable marker is a phleo selectable marker.

12. A retroviral packaging cell line according to any one of claims 10 or 11 wherein the packaging-deficient construct comprising the viral gag-pol gene and first selectable marker is the CeB (SEQ ID No 2) expression construct.

13. A retroviral packaging cell line according to any one of claims 10 or 11 wherein the packaging-deficient construct comprising the viral env gene and second selectable marker is the FBdelPASAF (SEQ ID No 5), the FBdelPMOSAF (SEQ ID No 6), the FbdelPGASAF (SEQ ID No 7), the FbdelPRDSAF (SEQ ID No 8), the FbdelPXSAF (Fig. 3), the FbdelP10A1SAF (Fig. 3), or the FBdelPVSVGSAF (Fig. 3) expression construct.

14. A retroviral packaging cell line according to any one of claims 10 or 11 wherein the recombinant expression vector is a packaging-deficient retroviral helper construct.

15. A retroviral packaging cell line according to claim 14 wherein the overlapping sequences between the genomes of the retroviral vector and the packaging-deficient construct is reduced by minimizing the extent of non-coding retroviral sequences in the packaging-deficient genome.

16. A retroviral packaging cell line according to any one of claims 10 to 15 wherein the viral gag-pol gene and the selectable marker are expressed under the control of a non-retroviral promoter.

17. A retroviral packaging cell line according to claim 16 wherein the promoter is fused to rabbit beta-1 globin intron.

18. A retroviral packaging cell line according to claim 16 or claim 17 wherein the promoter is a hCMV promoter.

19. A retroviral packaging cell line according to any one of claims 16 to claim 18 wherein the viral gag-pol gene and the selectable marker is a hCMV+intron (SEQ

ID No3) or a hCMV+intronkaSD (SEQ ID No 4) expression construct.

20. A retroviral packaging cell line according to anyone of claims 10 to 15 wherein the viral env gene and the selectable marker are under the control of a non-retroviral promoter.

21. A retroviral packaging cell line according to claim 20 wherein the promoter is fused to rabbit beta-1 globin intron.

22. A retroviral packaging cell line according to claim 20 or claim 21 wherein the promoter is a hCMV promoter.

23. A retroviral packaging cell line according any one of claims 20 to 22 wherein the viral env gene and the selectable marker is a CMV10A1 (SEQ ID No 9) expression construct.

24. A retroviral packaging cell line according to any one of claims 10 to 23 wherein the cell line is the HT1080 line, the TE671 line, the 3T3 line, the 293 line or the MV-1-lU line.

25. A retroviral packaging cell line according to anyone of claims 10 to 24 wherein the retroviral packaging cells comprises human HT1080 cells and express RD114 envelopes.

26. A retroviral packaging cell line according to anyone of claims 10 to 24 wherein the retroviral packaging cells comprises human TE671 cells and express RD114 envelopes.

27. A process for producing a retroviral packaging cell line in which a gene of interest in expressed, which process comprises:

transforming host cells with a first and a second recombinant expression vector, said first recombinant expression vector having a packaging-deficient construct comprising a viral gag-pol gene and a first selectable marker gene downstream thereof, and said second recombinant expression vector having a packaging-deficient construct comprising a viral env gene and a second selectable marker gene downstream thereof; wherein the start codon of the first and second selectable markers are spaced from the stop codons of the viral gag-pol gene and the viral env gene respectively by a distance which ensures that said selectable marker protein is expressed from the corresponding mRNA as a result of translation reinitiation;and

selecting transformed cells which express said first and/or second marker genes.

28. A packaging deficient construct for use in a process according to claim 27, which expresses a viral gag-pol gene and a selectable marker wherein a start codon of the selectable marker is spaced from a stop codon of the viral gag-pol gene by a distance which ensures that said selectable marker protein is expressed from the corresponding mRNA as a result of translation reinitiation.

29. A packaging deficient construct for use in a process according to claim 27, which expresses a viral env gene and a selectable marker gene; wherein a start codon of the selectable marker is spaced from a stop codon of the viral env gene by a distance which ensures that said selectable marker protein is

expressed from the corresponding mRNA as a result of translation reinitiation.

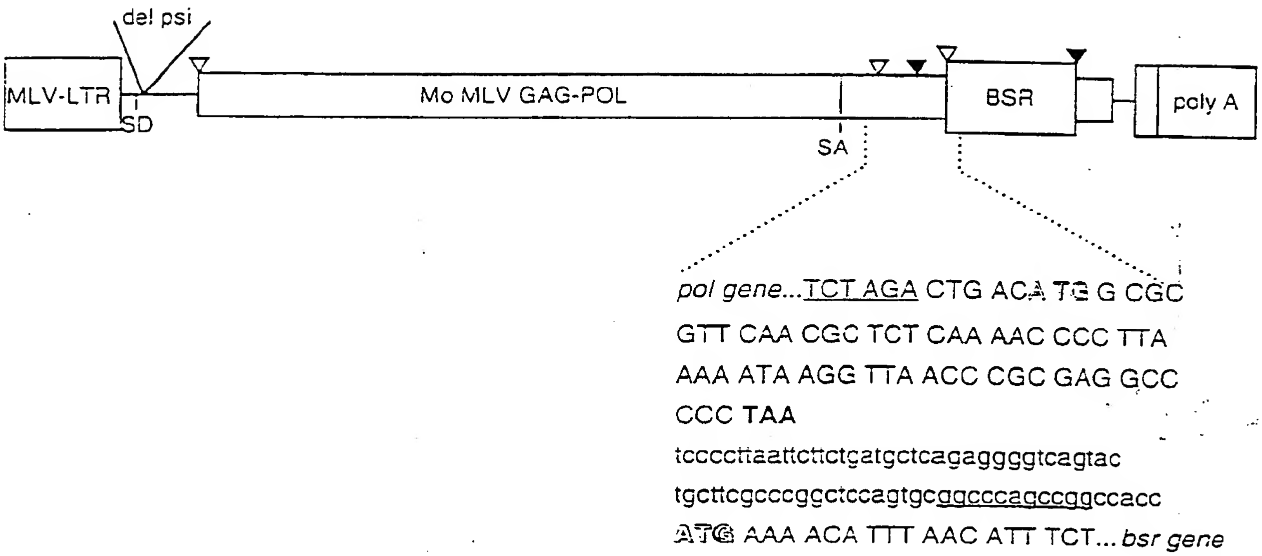
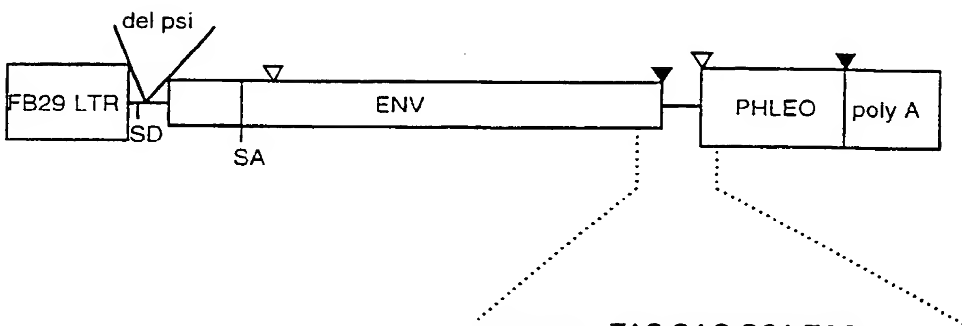


Figure 1. Schematic structure of CeB expression vector



env gene...TAC GAG CCA **TAG**
ggcgcc tagtgttgacaattaatcatcggcatagtata
cggcatagtataatacgactcactataggagggccacc
ATG GCC AAG TTG ACC...*phleo gene*

Figure 2. Schematic structure of FBdelPASF expression vector

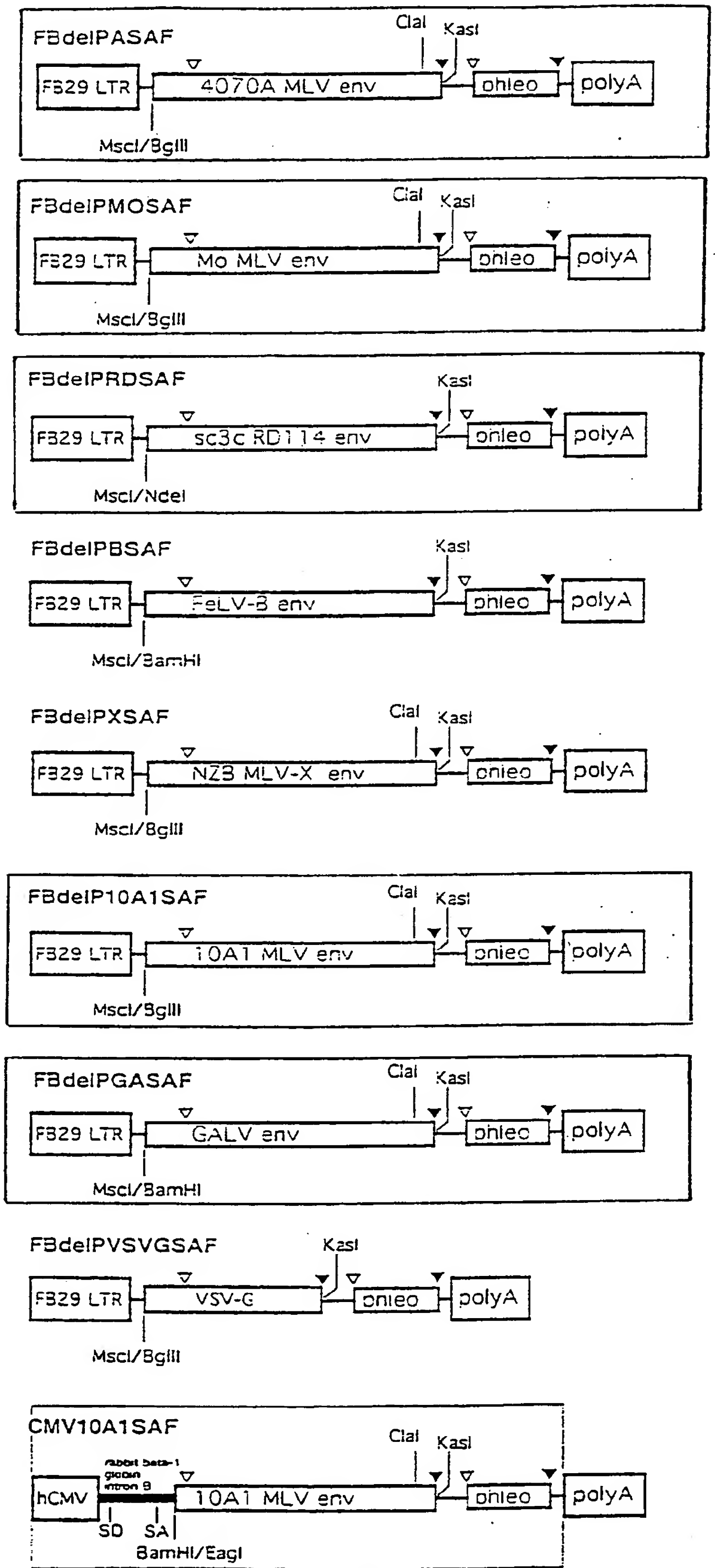


Figure 3. Schematic structure of env expression vectors

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GAAATACGCCCAACCGTTTTCGGCTCACCCCATATGAAATCCTTTATGGGGGACCCCCCC 180
CTTTGTCAACCTTGCTCAATTCCTTCTCCCCCTCCGATCCTAAGACTGATTTACAAGCCC 240
GACTAAAAGGGCTGCAAGGCGTGCAGGCCCAAATCTGGACACCCCTGGCCGAATTGTACC 300
GGCCAGGACATCCACAAACTAGCCACCCATTTCAGGTGGGAGACTCCGTGTACGTCCGGC 360
GGCACCGCTCTCAAGGATTGGAGCCTCGTTGGAAGGGACCTTACATCGTCCTGCTGACCA 420
CGCCCACCGCCATAAAGGTTGACGGGATCGCCGCCTGGATTCACGCATCGCACGCCAAGG 480
CAGCCCCAAAAACCCCTGGACCAGAAACTCCCAAAACCTGGAAGCTCCGCCGTTCGGAGA 540
ACCCTCTTAAGATAAGACTCTCCCGTGTCTGACTGCTAATCCACCTTGTCCCTGTACTAA 600
CCCAAAATGAAACTCCCAACAGGAATGGTCATTTTATGTAGCCTAATAATAGTTCGGGCA 660
GGGTTTGACGACCCCCGCAAGGCTATCGCATTAGTACAAAAACAACATGGTAAACCATGC 720
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CCAGGCAAGACGGCCTACTTAATGACCAACCAAAAATGGAAATGCAGAGTCACTCCAAAA 840
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CACAGTTCTTGTTATACTGAATACCGGCAATGCAGGCGAATTAATAAGACATACTACACG 960
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CTAGCAGACTCCCTAGCGAATGCCTCCTGTCAGATTATACCTCCCCTCTTGGTTCAACCG 1440
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GACTTAGGTGCAGTCACCTTTACTAACTGCACCTCTGTAGCCAATGTCAGTAGTCCTTTA 1560
TGTGCCCTAAACGGGTCAGTCTTCCTCTGTGGAAATAACATGGCATACACCTATTTACCC 1620
CAAAACTGGACCAGACTTTGCGTCCAAGCCTCCCTCCTCCCCGACATTGACATCAACCCG 1680
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GTAGTTCTCCAAAATAGGAGGGGACTGGACCTACTAACGGCAGAACAAGGAGGAATTTGT 1980
TTAGCCTTACAAGAAAAATGCTGTTTTTATGCTAACAAGTCAGGAATTGTGAGAAACAAA 2040
ATAAGAACCCTACAAGAAGAATTACAAAAACGCAGGGAAAGCCTGGCAACCAACCCTCTC 2100
TGGACCGGGCTGCAGGGCTTTCTTCCGTACCTCCTACCTCTCCTGGGACCCCTACTCACC 2160
CTCCTACTCATACTAACCATTGGGCCATGCGTTTTCAGTCGCCTCATGGCCTTCATTAAT 2220
GATAGACTTAATGTTGTACATGCCATGGTGCTGGCCCAGCAATACCAAGCACTCAAAGCT 2280
GAGGAAGAAGCTCAGGATTGAGCTTCCGGGACAAAAGCAGGGGGGAATGAGAAGTCAGAA 2340
CCCCCCACCTTTGCTACATAAATAACCGCTTTCATTTCGCTTCTGTAAAACGCTTATGCG 2400
CCCCACCCTAGCCGGAAAGTCCCCAGCCGCTACGCAACCCGGGCCCCGAGTTGCATCAGC 2460
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Fig.4

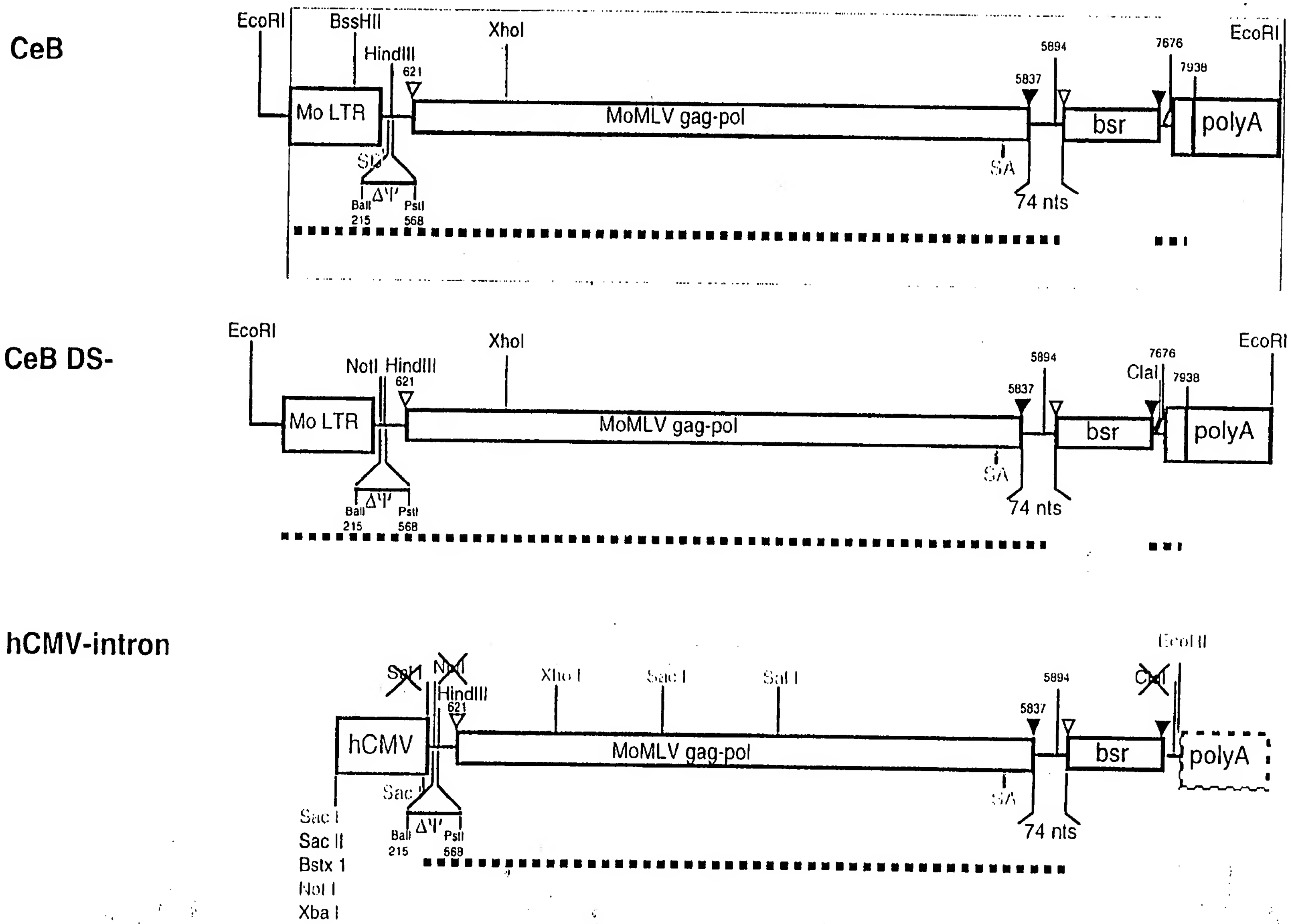


Figure 5. Genetic structure of gag-pol constructs (page 1/3)

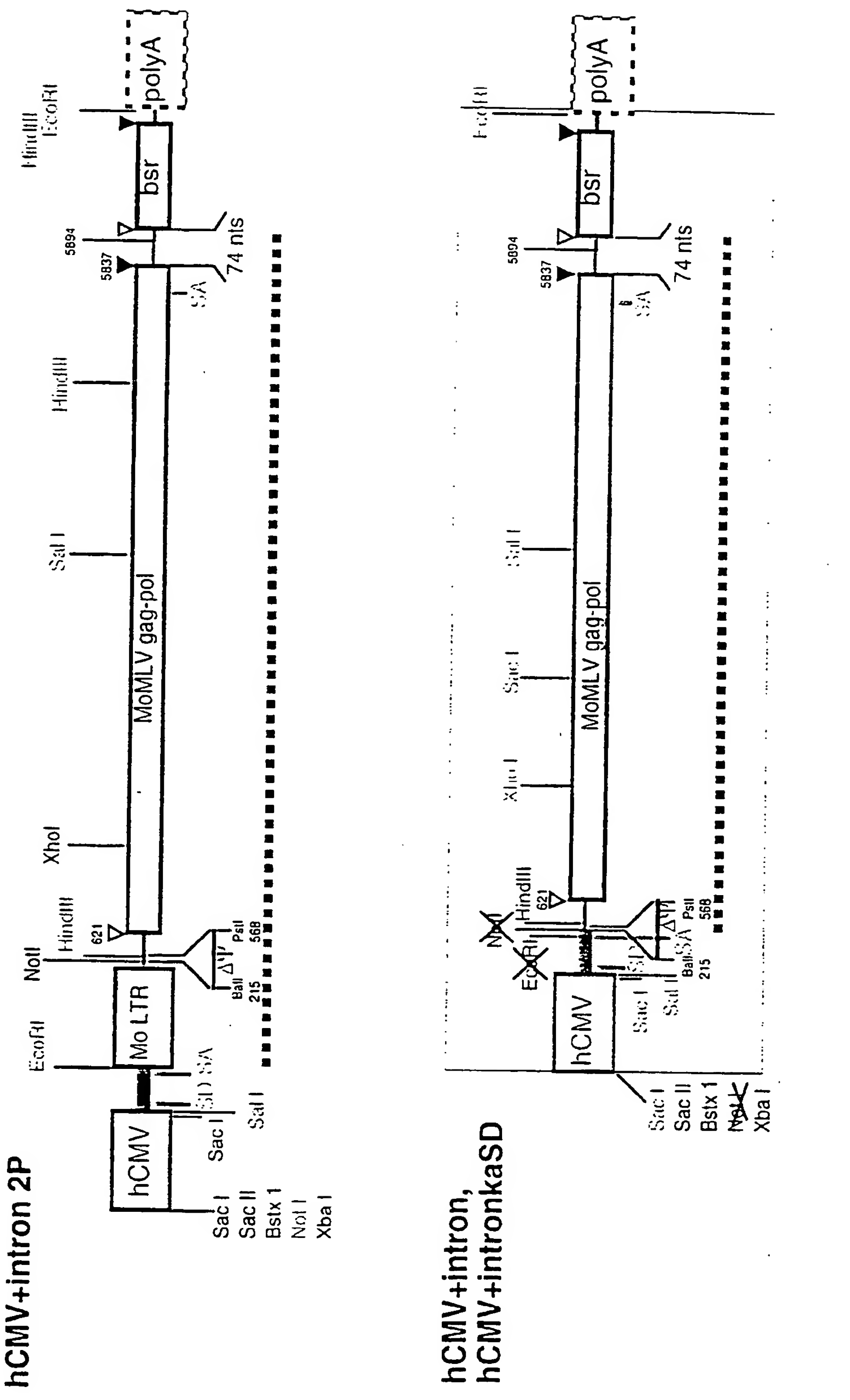
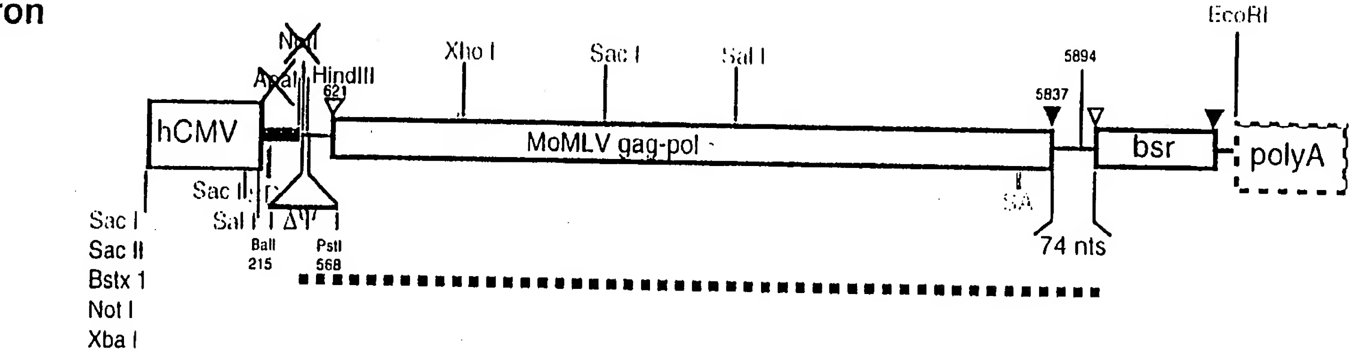


Figure 5. Genetic structure of gag-pol constructs (page 2/3)

hCMV+SD intron



hCMV+leader

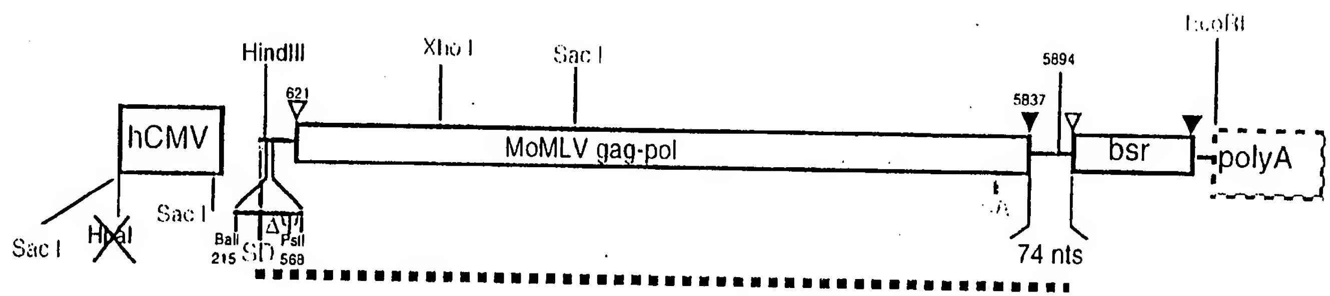


Figure 5. Genetic structure of gag-pol constructs (page 3/3)

Figure 6. CeB Sequence

1

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AGCTGAATAT GGGCCAAACA GGATATCTGT GGTAAGCAGT TCCTGCCCCG GCTCAGGGCC  180
AAGAACAGAT GGAACAGCTG AATATGGGCC AAACAGGATA TCTGTGGTAA GCAGTTCCTG  240
CCCCGGCTCA GGGCCAAGAA CAGATGGTCC CCAGATGCGG TCCAGCCCTC AGCAGTTTCT  300
AGAGAACCAT CAGATGTTTC CAGGGTGCCC CAAGGACCTG AAATGACCCT GTGCCTTATT  360
TGAACTAACC AATCAGTTCG CTTCTCGCTT CTGTTCGCGC GCTTCTGCTC CCCGAGCTCA  420
ATAAAAGAGC CCACAACCCC TCACTCGGGG CGCCAGTCCT CCGATTGACT GAGTCGCCCG  480
GGTACCCGTG TATCCAATAA ACCCTCTTGC AGTTGCATCC GACTTGTGGT CTCGCTGTTC  540
CTTGGGAGGG TCTCCTCTGA GTGATTGACT ACCCGTCAGC GGGGGTCTTT CATTTGGGGG  600
CTCGTCCGGG ATCGGGAGAC CCCTGCCCAG GGACCACCGA CCCACCACCG GGAGGTAAGC  660
TGGAAGCTTC TGCAGCATCG TTCTGTGTTG TCTCTGTCTG ACTGTGTTTC TGTATTTGTC  720
TGAGAATATG GGCCAGACTG TTACCACTCC CTTAAGTTTG ACCTTAGGTC ACTGGAAAGA  780
TGTCGAGCGG ATCGCTCACA ACCAGTCGGT AGATGTCAAG AAGAGACGTT GGGTTACCTT  840
CTGCTCTGCA GAATGGCCAA CCTTTAACGT CGGATGGCCG CGAGACGGCA CCTTTAACCG  900
AGACCTCATC ACCCAGGTTA AGATCAAGGT CTTTTCACCT GGCCCGCATG GACACCCAGA  960
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GCCCTTTGTA CACCCTAAGC CTCCGCCTCC TCTTCCTCCA TCCGCCCCGT CTCTCCCCCT 1080
TGAACCTCCT CGTTCGACCC CGCCTCGATC CTCCCTTTAT CCAGCCCTCA CTCCTTCTCT 1140
AGGCGCCAAA CCTAAACCTC AAGTTCTTTC TGACAGTGGG GGGCCGCTCA TCGACCTACT 1200
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ACGTGGGAGA CGGGAGCCCC CTGTGGCCGA CTCCACTACC TCGCAGGCAT TCCCCCTCCG 1380
CGCAGGAGGA AACGGACAGC TTCAATACTG GCCGTTCTCC TCTTCTGACC TTTACAACTG 1440
GAAAAATAAT AACCCTTCTT TTTCTGAAGA TCCAGGTAAA CTGACAGCTC TGATCGAGTC 1500
TGTTCTCATC ACCCATCAGC CCACCTGGGA CGACTGTCAG CAGCTGTTGG GGACTCTGCT 1560
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TGGGCGCCCC ACTCAACTGC CCAATGAAGT CGATGCCGCT TTTCCCCTCG AGCGCCCAGA 1680
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GGATGAGCAG AAAGAGAAAG AAAGAGATCG TAGGAGACAT AGAGAGATGA GCAAGCTATT 2160
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GTCCATAAAA CAATACCCCA TGTCACAAGA AGCCAGACTG GGGATCAAGC CCCACATACA 2940
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ACCCGTTAAG AAACCAGGGA CTAATGATTA TAGGCCTGTC CAGGATCTGA GAGAAGTCAA 3060
CAAGCGGGTG GAAGACATCC ACCCCACCGT GCCCAACCCT TACAACCTCT TGAGCGGGCT 3120
CCCACCGTCC CACCAGTGGT ACACTGTGCT TGATTTAAAG GATGCCTTTT TCTGCCTGAG 3180
ACTCCACCCC ACCAGTCAGC CTCTCTTCGC CTTTGAGTGG AGAGATCCAG AGATGGGAAT 3240
CTCAGGACAA TTGACCTGGA CCAGACTCCC ACAGGGTTTC AAAAACAGTC CCACCCTGTT 3300
TGATGAGGCA CTGCACAGAG ACCTAGCAGA CTTCCGGATC CAGCACCCAG ACTTGATCCT 3360
GCTACAGTAC GTGGATGACT TACTGCTGGC CGCCACTTCT GAGCTAGACT GCCAACAAGG 3420
TACTCGGGCC CTGTTACAAA CCCTAGGGAA CCTCGGGTAT CGGGCCTCGG CCAAGAAAGC 3480
CCAAATTTGC CAGAAACAGG TCAAGTATCT GGGGTATCTT CTAAAAGAGG GTCAGAGATG 3540
GCTGACTGAG GCCAGAAAAG AGACTGTGAT GGGGCAGCCT ACTCCGAAGA CCCCTCGACA 3600
ACTAAGGGAG TTCCTAGGGA CGGCAGGCTT CTGTCGCCTC TGGATCCCTG GGTTTGCAGA 3660
AATGGCAGCC CCCTTGTACC CTCTCACCAA AACGGGGACT CTGTTTAATT GGGGCCCAGA 3720
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GCCAGATTTG ACTAAGCCCT TTGAACTCTT TGTCGACGAG AAGCAGGGCT ACGCCAAAGG 3840
TGTCCTAACG CAAAAACTGG GACCTTGGCG TCGGCCGGTG GCCTACCTGT CCAAAAAGCT 3900
AGACCCAGTA GCAGCTGGGT GGCCCCCTTG CCTACGGATG GTAGCAGCCA TTGCCGTACT 3960
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AGTAGAGGCA CTAGTCAAAC AACCCCCCGA CCGCTGGCTT TCCAACGCCC GGATGACTCA 4080
```

Figure 6. CeB Sequence

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GGCCGAAGCC CACGGAACCC GACCCGACCT AACGGACCAG CCGCTCCCAG ACGCCGACCA 4260
CACCTGGTAC ACGGATGGAA GCAGTCTCTT ACAAGAGGGA CAGCGTAAGG CGGGAGCTGC 4320
GGTGACCACC GAGACCGAGG TAATCTGGGC TAAAGCCCTG CCAGCCGGGA CATCCGCTCA 4380
GCGGGCTGAA CTGATAGCAC TCACCCAGGC CCTAAAGATG GCAGAAGGTA AGAAGCTAAA 4440
TGTTTATACT GATAGCCGTT ATGCTTTTGC TACTGCCCAT ATCCATGGAG AAATATACAG 4500
AAGGCGTGGG TTGCTCACAT CAGAAGGCAA AGAGATCAAA AATAAAGACG AGATCTTGGC 4560
CCTACTAAAA GCCCTCTTTC TGCCCAAAAG ACTTAGCATA ATCCATTGTC CAGGACATCA 4620
AAAGGGACAC AGCGCCGAGG CTAGAGGCAA CCGGATGGCT GACCAAGCGG CCCGAAAGGC 4680
AGCCATCACA GAGACTCCAG ACACCTCTAC CCTCCTCATA GAAAATTCAT CACCCTACAC 4740
CTCAGAACAT TTTCATTACA CAGTGACTGA TATAAAGGAC CTAACCAAGT TGGGGGCCAT 4800
TTATGATAAA ACAAAGAAGT ATTGGGTCTA CCAAGGAAAA CCTGTGATGC CTGACCAGTT 4860
TACTTTTGAA TTATTAGACT TTCTTCATCA GCTGACTCAC CTCAGCTTCT CAAAAATGAA 4920
GGCTCTCCTA GAGAGAAGCC ACAGTCCCTA CTACATGCTG AACCGGGATC GAACACTCAA 4980
AAATATCACT GAGACCTGCA AAGCTTGTGC ACAAGTCAAC GCCAGCAAGT CTGCCGTTAA 5040
ACAGGGAACT AGGGTCCGCG GGCATCGGCC CGGCACTCAT TGGGAGATCG ATTTCACCGA 5100
GATAAAGCCC GGATTGTATG GCTATAAATA TCTTCTAGTT TTTATAGATA CCTTTTCTGG 5160
CTGGATAGAA GCCTTCCCAA CCAAGAAAGA AACCGCCAAG GTCGTAACCA AGAAGCTACT 5220
AGAGGAGATC TTCCCCAGGT TCGGCATGCC TCAGGTATTG GGAACTGACA ATGGGCCTGC 5280
CTTCGTCTCC AAGGTGAGTC AGACAGTGGC CGATCTGTTG GGGATTGATT GGAAATTACA 5340
TTGTGCATAC AGACCCCAAA GCTCAGGCCA GGTAGAAAGA ATGAATAGAA CCATCAAGGA 5400
GACTTTAACT AAATTAACGC TTGCAACTGG CTCTAGAGAC TGGGTGCTCC TACTCCCCTT 5460
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CAGCCCCTCT CTCCAAGCTC ACTTACAGGC TCTCTACTTA GTCCAGCACG AAGTCTGGAG 5640
ACCTCTGGCG GCAGCCTACC AAGAACAACT GGACCGACCG GTGGTACCTC ACCCTTACCG 5700
AGTCGGCGAC ACAGTGTGGG TCCGCCGACA CCAGACTAAG AACCTAGAAC CTCGCTGGAA 5760
AGGACCTTAC ACAGTCCTGC TGACCACCCC CACCGCCCTC AAAGTAGACG GCATCGCAGC 5820
TTGGATACAC GCCGCCCACG TGAAGGCTGC CGACCCCGGG GGTGGACCAT CCTCTAGACT 5880
GACATGGCGC GTTCAACGCT CTCAAAACCC CTTAAAAATA AGGTTAACCC GCGAGGCCCC 5940
CTAATCCCCT TAATTCTTCT GATGCTCAGA GGGGTCAGTA CTGCTTCGCC CGGCTCCAGT 6000
GCGGCCCAGC CGGCCACCAT GAAAACATTT AACATTTCTC AACAAGATCT AGAATTAGTA 6060
GAAGTAGCGA CAGAGAAGAT TACAATGCTT TATGAGGATA ATAAACATCA TGTGGGAGCG 6120
GCAATTCGTA CGAAAACAGG AGAAATCATT TCGGCAGTAC ATATTGAAGC GTATATAGGA 6180
CGAGTAACTG TTTGTGCAGA AGCCATTGCG ATTGGTAGTG CAGTTTCGAA TGGACAAAAG 6240
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GTTCATCTGC TGACTGTCAA CTGTAGCATT TTTTGGGGTT ACAGTTTGAG CAGGATATTT 7380
GGTCCTGTAG TTTGCTAACA CACCCTGCAG CTCCAAAGGT TCCCCACCAA CAGCAAAAAA 7440
ATGAAAATTT GACCCTTGAA TGGGTTTTCC AGCACCATTT TCATGAGTTT TTTGTGTCCC 7500
TGAATGCAAG TTTAACATAG CAGTTACCCC AATAACCTCA GTTTTAACAG TAACAGCTTC 7560
CCACATCAAA ATATTTCCAC AGGTTAAGTC CTCATTTAAA TTAGGCAAAG GAATTC 7616

Figure 7. hCMV+intron Sequence 1

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AGATCTCCCG ATCCCCTATG GTCGACTCTC AGTACAATCT GCTCTGATGC CGCATAGTTA     60
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CGTTTTGCGC TGCTTCGCGA TGTACGGGCC AGATATACGC GTTGACATTG ATTATTGACT    240
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ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA TTGACGTCAA    420
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AGTACGCCCC CTATTGACGT CAATGACGGT AAATGGCCCG CCTGGCATTA TGCCCAGTAC    540
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ACAGCTCCTG GGCAACGTGC TGGTTGTTGT GCTGTCTCAT CATTTTGGCA AGAATTGGCC   1500
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CAGGTCCCCT ACATCGTGAC CTGGGAAGCC TTGGCTTTTG ACCCCCCTCC CTGGGTCAAG   1860
CCCTTTGTAC ACCCTAAGCC TCCGCCTCCT CTTCCTCCAT CCGCCCCGTC TCTCCCCCTT   1920
GAACCTCCTC GTTCGACCCC GCCTCGATCC TCCCTTTATC CAGCCCTCAC TCCTTCTCTA   1980
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Figure 7. hCMV+intron Sequence 2

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CAAATTTGCC AGAAACAGGT CAAGTATCTG GGGTATCTTC TAAAAGAGGG TCAGAGATGG 4380
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GAGTAACTGT TTGTGCAGAA GCCATTGCGA TTGGTAGTGC AGTTTCGAAT GGACAAAAGG 7080
ATTTTGACAC GATTGTAGCT GTTAGACACC CTTATTCTGA CGAAGTAGAT AGAAGTATTC 7140
GAGTGGTAAG TCCTTGTGGT ATGTGTAGGG AGTTGATTTC AGACTATGCA CCAGATTGTT 7200
TTGTGTTAAT AGAAATGAAT GGCAAGTTAG TCAAAACTAC GATTGAAGAA CTCATTCCAC 7260
TCAAATATAC CCGAAATTAA AAGTTTTACC ACCAAGCTTA TCGAATTC 7308
```

Figure 8. hCMV+intronkaSD Sequence 1

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AGATCTCCCG ATCCCCTATG GTCGACTCTC AGTACAATCT GCTCTGATGC CGCATAGTTA   60
AGCCAGTATC TGCTCCCTGC TTGTGTGTTG GAGGTCGCTG AGTAGTGCGC GAGCAAAATT  120
TAAGCTACAA CAAGGCAAGG CTTGACCGAC AATTGCATGA AGAATCTGCT TAGGGTTAGG  180
CGTTTTGCGC TGCTTCGCGA TGTACGGGCC AGATATACGC GTTGACATTG ATTATTGACT  240
AGTTATTAAT AGTAATCAAT TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC  300
GTTACATAAC TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG  360
ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA TTGACGTCAA  420
TGGGTGGACT ATTTACGGTA AACTGCCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA  480
AGTACGCCCC CTATTGACGT CAATGACGGT AAATGGCCCG CCTGGCATTA TGCCCAGTAC  540
ATGACCTTAT GGGACTTTCC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC  600
ATGGTGATGC GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA  660
TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA AAATCAACGG  720
GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA  780
CGGTGGGAGG TCTATATAAG CAGAGCTCTC TGGCTAACTA GAGAACCCAC TGCTTAACTG  840
GCTTATCGAA ATGTCGACTG AGAACTTCAG GGTGAGTTTG GGGACCCTTG ATTGTTCTTT  900
CTTTTTCGCT ATTGTAAAAT TCATGTTATA TGGAGGGGGC AAAGTTTTCA GGGTGTTGTT  960
TAGAATGGGA AGATGTCCCT TGTATCACCA TGGACCCTCA TGATAATTTT GTTTCTTTCA 1020
CTTTCTACTC TGTTGACAAC CATTGTCTCC TCTTATTTTC TTTTCATTTT CTGTAACTTT 1080
TTCGTTAAAC TTTAGCTTGC ATTTGTAACG AATTTTTAAA TTCACTTTTG TTTATTTGTC 1140
AGATTGTAAG TACTTTCTCT AATCACTTTT TTTTCAAGGC AATCAGGGTA TATTATATTG 1200
TACTTCAGCA CAGTTTTAGA GAACAATTGT TATAATTAAA TGATAAGGTA GAATATTTCT 1260
GCATATAAAT TCTGGCTGGC GTGGAAATAT TCTTATTGGT AGAAACAACT ACATCCTGGT 1320
CATCATCCTG CCTTTCTCTT TATGGTTACA ATGATATACA CTGTTTGAGA TGAGGATAAA 1380
ATACTCTGAG TCCAAACCGG GCCCCTCTGC TAACCATGTT CATGCCTTCT TCTTTTTCCT 1440
ACAGCTCCTG GGCAACGTGC TGGTTGTTGT GCTGTCTCAT CATTTTGGCA AGAATTGGCC 1500
GCAAGCTTCT GCAGCATCGT TCTGTGTTGT CTCTGTCTGA CTGTGTTTCT GTATTTGTCT 1560
GAGAATATGG GCCAGACTGT TACCACTCCC TTAAGTTTGA CCTTAGGTCA CTGGAAAGAT 1620
GTCGAGCGGA TCGCTCACAA CCAGTCGGTA GATGTCAAGA AGAGACGTTG GGTTACCTTC 1680
TGCTCTGCAG AATGGCCAAC CTTTAACGTC GGATGGCCGC GAGACGGCAC CTTTAACCGA 1740
GACCTCATCA CCCAGGTTAA GATCAAGGTC TTTTCACCTG GCCCGCATGG ACACCCAGAC 1800
CAGGTCCCCT ACATCGTGAC CTGGGAAGCC TTGGCTTTTG ACCCCCCTCC CTGGGTCAAG 1860
CCCTTTGTAC ACCCTAAGCC TCCGCCTCCT CTTCCTCCAT CCGCCCCGTC TCTCCCCCTT 1920
GAACCTCCTC GTTCGACCCC GCCTCGATCC TCCCTTTATC CAGCCCTCAC TCCTTCTCTA 1980
GGCGCCAAAC CTAAACCTCA AGTTCTTTCT GACAGTGGGG GGCCGCTCAT CGACCTACTT 2040
ACAGAAGACC CCCCGCCTTA TAGGGACCCA AGACCACCCC CTTCCGACAG GGACGGAAAT 2100
GGTGGAGAAG CGACCCCTGC GGGAGAGGCA CCGGACCCCT CCCCAATGGC ATCTCGCCTA 2160
CGTGGGAGAC GGGAGCCCCC TGTGGCCGAC TCCACTACCT CGCAGGCATT CCCCCTCCGC 2220
GCAGGAGGAA ACGGACAGCT TCAATACTGG CCGTTCTCCT CTTCTGACCT TTACAACTGG 2280
AAAAATAATA ACCCTTCTTT TTCTGAAGAT CCAGGTAAAC TGACAGCTCT GATCGAGTCT 2340
GTTCTCATCA CCCATCAGCC CACCTGGGAC GACTGTCAGC AGCTGTTGGG GACTCTGCTG 2400
ACCGGAGAAG AAAAACAACG GGTGCTCTTA GAGGCTAGAA AGGCGGTGCG GGGCGATGAT 2460
GGGCGCCCCA CTCAACTGCC CAATGAAGTC GATGCCGCTT TTCCCCTCGA GCGCCCAGAC 2520
TGGGATTACA CCACCCAGGC AGGACGCAAC CACCTAGTCC ACTATCGCCA GTTGCTCCTA 2580
GCGGGTCTCC AAAACGCGGG CAGAAGCCCC ACCAATTTGG CCAAGGTAAA AGGAATAACA 2640
CAAGGGCCCA ATGAGTCTCC CTCGGCCTTC CTAGAGAGAC TTAAGGAAGC CTATCGCAGG 2700
TACACTCCTT ATGACCCTGA GGACCCAGGG CAAGAAACTA ATGTGTCTAT GTCTTTCATT 2760
TGGCAGTCTG CCCCAGACAT TGGGAGAAAG TTAGAGAGGT TAGAAGATTT AAAAAACAAG 2820
ACGCTTGGAG ATTTGGTTAG AGAGGCAGAA AAGATCTTTA ATAAACGAGA AACCCCGGAA 2880
GAAAGAGAGG AACGTATCAG GAGAGAAACA GAGGAAAAAG AAGAACGCCG TAGGACAGAG 2940
GATGAGCAGA AAGAGAAAGA AAGAGATCGT AGGAGACATA GAGAGATGAG CAAGCTATTG 3000
GCCACTGTCG TTAGTGGACA GAAACAGGAT AGACAGGGAG GAGAACGAAG GAGGTCCCAA 3060
CTCGATCGCG ACCAGTGTGC CTACTGCAAA GAAAAGGGGC ACTGGGCTAA AGATTGTCCC 3120
AAGAAACCAC GAGGACCTCG GGGACCAAGA CCCCAGACCT CCCTCCTGAC CCTAGATGAC 3180
TAGGGAGGTC AGGGTCAGGA GCCCCCCCCT GAACCCAGGA TAACCCTCAA AGTCGGGGGG 3240
CAACCCGTCA CCTTCCTGGT AGATACTGGG GCCCAACACT CCGTGCTGAC CCAAAATCCT 3300
GGACCCCTAA GTGATAAGTC TGCCTGGGTC CAAGGGGCTA CTGGAGGAAA GCGGTATCGC 3360
TGGACCACGG ATCGCAAAGT ACATCTAGCT ACCGGTAAGG TCACCCACTC TTTCCTCCAT 3420
GTACCAGACT GTCCCTATCC TCTGTTAGGA AGAGATTTGC TGACTAAACT AAAAGCCCAA 3480
ATCCACTTTG AGGGATCAGG AGCTCAGGTT ATGGGACCAA TGGGGCAGCC CCTGCAAGTG 3540
TTGACCCTAA ATATAGAAGA TGAGCATCGG CTACATGAGA CCTCAAAAGA GCCAGATGTT 3600
TCTCTAGGGT CCACATGGCT GTCTGATTTT CCTCAGGCCT GGGCGGAAAC CGGGGGCATG 3660
GGACTGGCAG TTCGCCAAGC TCCTCTGATC ATACCTCTGA AAGCAACCTC TACCCCCGTG 3720
TCCATAAAAC AATACCCCAT GTCACAAGAA GCCAGACTGG GGATCAAGCC CCACATACAG 3780
AGACTGTTGG ACCAGGGAAT ACTGGTACCC TGCCAGTCCC CCTGGAACAC GCCCCTGCTA 3840
CCCGTTAAGA AACCAGGGAC TAATGATTAT AGGCCTGTCC AGGATCTGAG AGAAGTCAAC 3900
AAGCGGGTGG AAGACATCCA CCCCACCGTG CCCAACCCTT ACAACCTCTT GAGCGGGCTC 3960
CCACCGTCCC ACCAGTGGTA CACTGTGCTT GATTTAAAGG ATGCCTTTTT CTGCCTGAGA 4020
CTCCACCCCA CCAGTCAGCC TCTCTTCGCC TTTGAGTGGA GAGATCCAGA GATGGGAATC 4080
```

Figure 8. hCMV+intronkaSD Sequence 2

TCAGGACAAT TGACCTGGAC CAGACTCCCA CAGGGTTTCA AAAACAGTCC CACCCTGTTT 4140
GATGAGGCAC TGCACAGAGA CCTAGCAGAC TTCCGGATCC AGCACCCAGA CTTGATCCTG 4200
CTACAGTACG TGGATGACTT ACTGCTGGCC GCCACTTCTG AGCTAGACTG CCAACAAGGT 4260
ACTCGGGCCC TGTTACAAAC CCTAGGGAAC CTCGGGTATC GGGCCTCGGC CAAGAAAGCC 4320
CAAATTTGCC AGAAACAGGT CAAGTATCTG GGGTATCTTC TAAAAGAGGG TCAGAGATGG 4380
CTGACTGAGG CCAGAAAAGA GACTGTGATG GGGCAGCCTA CTCCGAAGAC CCCTCGACAA 4440
CTAAGGGAGT TCCTAGGGAC GGCAGGCTTC TGTCGCCTCT GGATCCCTGG GTTTGCAGAA 4500
ATGGCAGCCC CCTTGTACCC TCTCACCAAA ACGGGGACTC TGTTTAATTG GGGCCCAGAC 4560
CAACAAAAGG CCTATCAAGA AATCAAGCAA GCTCTTCTAA CTGCCCCAGC CCTGGGGTTG 4620
CCAGATTTGA CTAAGCCCTT TGAACTCTTT GTCGACGAGA AGCAGGGCTA CGCCAAAGGT 4680
GTCCTAACGC AAAAACTGGG ACCTTGGCGT CGGCCGGTGG CCTACCTGTC CAAAAAGCTA 4740
GACCCAGTAG CAGCTGGGTG GCCCCCTTGC CTACGGATGG TAGCAGCCAT TGCCGTACTG 4800
ACAAAGGATG CAGGCAAGCT AACCATGGGA CAGCCACTAG TCATTCTGGC CCCCCATGCA 4860
GTAGAGGCAC TAGTCAAACA ACCCCCCGAC CGCTGGCTTT CCAACGCCCG GATGACTCAC 4920
TATCAGGCCT TGCTTTTGGA CACGGACCGG GTCCAGTTCG GACCGGTGGT AGCCCTGAAC 4980
CCGGCTACGC TGCTCCCACT GCCTGAGGAA GGGCTGCAAC ACAACTGCCT TGATATCCTG 5040
GCCGAAGCCC ACGGAACCCG ACCCGACCTA ACGGACCAGC CGCTCCCAGA CGCCGACCAC 5100
ACCTGGTACA CGGATGGAAG CAGTCTCTTA CAAGAGGGAC AGCGTAAGGC GGGAGCTGCG 5160
GTGACCACCG AGACCGAGGT AATCTGGGCT AAAGCCCTGC CAGCCGGGAC ATCCGCTCAG 5220
CGGGCTGAAC TGATAGCACT CACCCAGGCC CTAAAGATGG CAGAAGGTAA GAAGCTAAAT 5280
GTTTATACTG ATAGCCGTTA TGCTTTTGCT ACTGCCCATA TCCATGGAGA AATATACAGA 5340
AGGCGTGGGT TGCTCACATC AGAAGGCAAA GAGATCAAAA ATAAAGACGA GATCTTGGCC 5400
CTACTAAAAG CCCTCTTTCT GCCCAAAAGA CTTAGCATAA TCCATTGTCC AGGACATCAA 5460
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GCCATCACAG AGACTCCAGA CACCTCTACC CTCCTCATAG AAAATTCATC ACCCTACACC 5580
TCAGAACATT TTCATTACAC AGTGACTGAT ATAAAGGACC TAACCAAGTT GGGGGCCATT 5640
TATGATAAAA CAAAGAAGTA TTGGGTCTAC CAAGGAAAAC CTGTGATGCC TGACCAGTTT 5700
ACTTTTGAAT TATTAGACTT TCTTCATCAG CTGACTCACC TCAGCTTCTC AAAAATGAAG 5760
GCTCTCCTAG AGAGAAGCCA CAGTCCCTAC TACATGCTGA ACCGGGATCG AACACTCAAA 5820
AATATCACTG AGACCTGCAA AGCTTGTGCA CAAGTCAACG CCAGCAAGTC TGCCGTTAAA 5880
CAGGGAACTA GGGTCCGCGG GCATCGGCCC GGCACTCATT GGGAGATCGA TTTCACCGAG 5940
ATAAAGCCCG GATTGTATGG CTATAAATAT CTTCTAGTTT TTATAGATAC CTTTTCTGGC 6000
TGGATAGAAG CCTTCCCAAC CAAGAAAGAA ACCGCCAAGG TCGTAACCAA GAAGCTACTA 6060
GAGGAGATCT TCCCCAGGTT CGGCATGCCT CAGGTATTGG GAACTGACAA TGGGCCTGCC 6120
TTCGTCTCCA AGGTGAGTCA GACAGTGGCC GATCTGTTGG GGATTGATTG GAAATTACAT 6180
TGTGCATACA GACCCCAAAG CTCAGGCCAG GTAGAAAGAA TGAATAGAAC CATCAAGGAG 6240
ACTTTAACTA AATTAACGCT TGCAACTGGC TCTAGAGACT GGGTGCTCCT ACTCCCCTTA 6300
GCCCTGTACC GAGCCCGCAA CACGCCGGGC CCCCATGGCC TCACCCCATA TGAGATCTTA 6360
TATGGGGCAC CCCCGCCCCT TGTAAACTTC CCTGACCCTG ACATGACAAG AGTTACTAAC 6420
AGCCCCTCTC TCCAAGCTCA CTTACAGGCT CTCTACTTAG TCCAGCACGA AGTCTGGAGA 6480
CCTCTGGCGG CAGCCTACCA AGAACAACTG GACCGACCGG TGGTACCTCA CCCTTACCGA 6540
GTCGGCGACA CAGTGTGGGT CCGCCGACAC CAGACTAAGA ACCTAGAACC TCGCTGGAAA 6600
GGACCTTACA CAGTCCTGCT GACCACCCCC ACCGCCCTCA AAGTAGACGG CATCGCAGCT 6660
TGGATACACG CCGCCCACGT GAAGGCTGCC GACCCCGGGG GTGGACCATC CTCTAGACTG 6720
ACATGGCGCG TTCAACGCTC TCAAAACCCC TTAAAAATAA GGTTAACCCG CGAGGCCCCC 6780
TAATCCCCTT AATTCTTCTG ATGCTCAGAG GGGTCAGTAC TGCTTCGCCC GGCTCCAGTG 6840
CGGCCCAGCC GGCCACCATG AAAACATTTA ACATTTCTCA ACAAGATCTA GAATTAGTAG 6900
AAGTAGCGAC AGAGAAGATT ACAATGCTTT ATGAGGATAA TAAACATCAT GTGGGAGCGG 6960
CAATTCGTAC GAAAACAGGA GAAATCATTT CGGCAGTACA TATTGAAGCG TATATAGGAC 7020
GAGTAACTGT TTGTGCAGAA GCCATTGCGA TTGGTAGTGC AGTTTCGAAT GGACAAAAGG 7080
ATTTTGACAC GATTGTAGCT GTTAGACACC CTTATTCTGA CGAAGTAGAT AGAAGTATTC 7140
GAGTGGTAAG TCCTTGTGGT ATGTGTAGGG AGTTGATTTC AGACTATGCA CCAGATTGTT 7200
TTGTGTTAAT AGAAATGAAT GGCAAGTTAG TCAAAACTAC GATTGAAGAA CTCATTCCAC 7260
TCAAATATAC CCGAAATTAA AAGTTTTACC ACCAAGCTTA TCGAATTC 7308

Figure 9. FBdelPASAF Sequence 1

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CATATGCGGT GTGAAATACC GCACAGATGC GTAAGGAGAA AATACCGCAT CAGGCGCCAT     60
TCGCCATTCA GGCTGCGCAA CTGTTGGGAA GGGCGATCGG TGCGGGCCTC TTCGCTATTA    120
CGCCAGCTGG CGAAAGGGGG ATGTGCTGCA AGGCGATTAA GTTGGGTAAC GCCAGGGTTT    180
TCCCAGTCAC GACGTTGTAA AACGACGGCC AGTGAATTCC GATTAGTTCA ATTTGTTAAA    240
GACAGGATCT CAGTAGTCCA GGCTTTAGTC CTGACTCAAC AATACCACCA GCTAAAACCA    300
CTAGAATACG AGCCACAATA AATAAAAGAT TTTATTTAGT TTCCAGAAAA AGGGGGGAAT    360
GAAAGACCCC ACCAAATTGC TTAGCCTGAT AGCCGCAGTA ACGCCATTTT GCAAGGCATG    420
GAAAAATACC AAACCAAGAA TAGAGAAGTT CAGATCAAGG GCGGGTACAC GAAAACAGCT    480
AACGTTGGGC CAAACAGGAT ATCTGCGGTG AGCAGTTTCG GCCCCGGCCC GGGGCCAAGA    540
ACAGATGGTC ACCGCGGTTC GGCCCCGGCC CGGGGCCAAG AACAGATGGT CCCCAGATAT    600
GGCCCAACCC TCAGCAGTTT CTTAAGACCC ATCAGATGTT TCCAGGCTCC CCCAAGGACC    660
TGAAATGACC CTGTGCCTTA TTTGAATTAA CCAATCAGCC TGCTTCTCGC TTCTGTTCGC    720
GCGCTTCTGC TTCCCGAGCT CTATAAAAGA GCTCACAACC CCTCACTCGG CGCGCCAGTC    780
CTCCGATAGA CTGAGTCGCC CGGGTACCCG TGTATCCAAT AAATCCTCTT GCTGTTGCAT    840
CCGACTCGTG GTCTCGCTGT TCCTTGGGAG GGTCTCCTCA GAGTGATTGA CTACCCGTCT    900
CGGGGGTCTT TCATTTGGGG GCTCGTCCGG GATCTGGAGA CCCCTGCCCA GGGACCACCG    960
ACCCACCACC GGGAGGTAAG CTGGCCAAGA TCTTATATGG GGCACCCCCG CCCCTTGTAA   1020
ACTTCCCTGA CCCTGACATG ACCAGAGTTA CTAACAGCCC CTCTCTCCAA GCTCACTTAC   1080
AGGCTCTCTA CTTAGTCCAG CACGAAGTTT GGAGACCACT GGCGGCAGCT TACCAAGAAC   1140
AACTGGACCG GCCGGTGGTG CCTCACCCTT ACCGGGTCGG CGACACAGTG TGGGTCCGCC   1200
GACATCAAAC CAAGAACCTA GAACCTCGCT GGAAAGGACC TTACACAGTC CTGCTGACCA   1260
CCCCCACCGC CCTCAAAGTA GACGGTATCG CAGCTTGGAT ACACGCAGCC CACGTAAAGG   1320
CGGCCGACAC CGAGAGTGGA CCATCCTCTG GACGGACATG GCGCGTTCAA CGCTCTCAAA   1380
ACCCCCTCAA GATAAGATTA ACCCGTGGAA GCCCTTAATA GTCATGGGAG TCCTGTTAGG   1440
AGTAGGGATG GCAGAGAGCC CCCATCAGGT CTTTAATGTA ACCTGGAGAG TCACCAACCT   1500
GATGACTGGG CGTACCGCCA ATGCCACCTC CCTCCTGGGA ACTGTACAAG ATGCCTTCCC   1560
AAAATTATAT TTTGATCTAT GTGATCTGGT CGGAGAGGAG TGGGACCCTT CAGACCAGGA   1620
ACCGTATGTC GGGTATGGCT GCAAGTACCC CGCAGGGAGA CAGCGGACCC GGACTTTTGA   1680
CTTTTACGTG TGCCCTGGGC ATACCGTAAA GTCGGGGTGT GGGGGACCAG GAGAGGGCTA   1740
CTGTGGTAAA TGGGGGTGTG AAACCACCGG ACAGGCTTAC TGGAAGCCCA CATCATCGTG   1800
GGACCTAATC TCCCTTAAGC GCGGTAACAC CCCCTGGGAC ACGGGATGCT CTAAAGTTGC   1860
CTGTGGCCCC TGCTACGACC TCTCCAAAGT ATCCAATTCC TTCCAAGGGG CTACTCGAGG   1920
GGGCAGATGC AACCCTCTAG TCCTAGAATT CACTGATGCA GGAAAAAAGG CTAACTGGGA   1980
CGGGCCCAAA TCGTGGGGAC TGAGACTGTA CCGGACAGGA ACAGATCCTA TTACCATGTT   2040
CTCCCTGACC CGGCAGGTCC TTAATGTGGG ACCCCGAGTC CCCATAGGGC CCAACCCAGT   2100
ATTACCCGAC CAAAGACTCC CTTCCTCACC AATAGAGATT GTACCGGCTC CACAGCCACC   2160
TAGCCCCCTC AATACCAGTT ACCCCCCTTC CACTACCAGT ACACCCTCAA CCTCCCCTAC   2220
AAGTCCAAGT GTCCCACAGC CACCCCCAGG AACTGGAGAT AGACTACTAG CTCTAGTCAA   2280
AGGAGCCTAT CAGGCGCTTA ACCTCACCAA TCCCGACAAG ACCCAAGAAT GTTGGCTGTG   2340
CTTAGTGTCG GGACCTCCTT ATTACGAAGG AGTAGCGGTC GTGGGCACTT ATACCAATCA   2400
TTCCACCGCT CCGGCCAACT GTACGGCCAC TTCCCAACAT AAGCTTACCC TATCTGAAGT   2460
GACAGGACAG GGCCTATGCA TGGGGGCAGT ACCTAAAACT CACCAGGCCT TATGTAACAC   2520
CACCCAAAGC GCCGGCTCAG GATCCTACTA CCTTGCAGCA CCCGCCGGAA CAATGTGGGC   2580
TTGCAGCACT GGATTGACTC CCTGCTTGTC CACCACGGTG CTCAATCTAA CCACAGATTA   2640
TTGTGTATTA GTTGAACTCT GGCCCAGAGT AATTTACCAC TCCCCCGATT ATATGTATGG   2700
TCAGCTTGAA CAGCGTACCA AATATAAAAG AGAGCCAGTA TCATTGACCC TGGCCCTTCT   2760
ACTAGGAGGA TTAACCATGG GAGGGATTGC AGCTGGAATA GGGACGGGGA CCACTGCCTT   2820
AATTAAAACC CAGCAGTTTG AGCAGCTTCA TGCCGCTATC CAGACAGACC TCAACGAAGT   2880
CGAAAAGTCA ATTACCAACC TAGAAAAGTC ACTGACCTCG TTGTCTGAAG TAGTCCTACA   2940
GAACCGCAGA GGCCTAGATT TGCTATTCCT AAAGGAGGGA GGTCTCTGCG CAGCCCTAAA   3000
AGAAGAATGT TGTTTTTATG CAGACCACAC GGGGCTAGTG AGAGACAGCA TGGCCAAATT   3060
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GCTGTTTAAT AGATCCCCCT GGTTTACCAC CTTAATCTCC ACCATCATGG GACCTCTAAT   3180
AGTACTCTTA CTGATCTTAC TCTTTGGACC TTGCATTCTC AATCGATTAG TTCAATTTGT   3240
TAAAGACAGG ATCTCAGTAG TCCAGGCTTT AGTCCTGACT CAACAATACC ACCAGCTAAA   3300
GCCTATAGAG TACGAGCCAT AGGGCGCCTA GTGTTGACAA TTAATCATCG GCATAGTATA   3360
CGGCATAGTA TAATACGACT CACTATAGGA GGGCCACCAT GGCCAAGTTG ACCAGTGCCG   3420
TTCCGGTGCT CACCGCGCGC GACGTCGCCG GAGCGGTCGA GTTCTGGACC GACCGGCTCG   3480
GGTTCTCCCG GGACTTCGTG GAGGACGACT TCGCCGGTGT GGTCCGGGAC GACGTGACCC   3540
TGTTCATCAG CGCGGTCCAG GACCAGGTGG TGCCGGACAA CACCCTGGCC TGGGTGTGGG   3600
TGCGCGGCCT GGACGAGCTG TACGCCGAGT GGTCGGAGGT CGTGTCCACG AACTTCCGGG   3660
ACGCCTCCGG GCCGGCCATG ACCGAGATCG GCGAGCAGCC GTGGGGGCGG GAGTTCGCCC   3720
TGCGCGACCC GGCCGGCAAC TGCGTGCACT TCGTGGCCGA GGAGCAGGAC TGANNNNCGG   3780
ACCGGTCGAC TTGTTAACTT GTTTATTGCA GCTTATAATG GTTACAAATA AAGCAATAGC   3840
ATCACAAATT TCACAAATAA AGCATTTTTT TCACTGCATT CTAGTTGTGG TTTGTCCAAA   3900
CTCATCAATG TATCTTATCA TGTCTGGATC CAGATCTGGG CCCATGCGGC CGCGGATCGA   3960
TNNNNACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT   4020
GGCGTTTTTC CATAGGCTCC GCCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA   4080
```

Figure 9. FBdelPASAF Sequence 2

GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT 4140
CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC 4200
GGGAAGCGTG GCGCTTTCTC AATGCTCACG CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT 4260
TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG CCCGACCGCT GCGCCTTATC 4320
CGGTAACTAT CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC 4380
CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG 4440
GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT ATCTGCGCTC TGCTGAAGCC 4500
AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA CCGCTGGTAG 4560
CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA AAAAAAGGAT CTCAAGAAGA 4620
TCCTTTGATC TTTTCTACGG GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT 4680
TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC CTTTTAAATT AAAAATGAAG 4740
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CAGTGAGGCA CCTATCTCAG CGATCTGTCT ATTTCGTTCA TCCATAGTTG CCTGACTCCC 4860
CGTCGTGTAG ATAACTACGA TACGGGAGGG CTTACCATCT GGCCCCAGTG CTGCAATGAT 4920
ACCGCGAGAC CCACGCTCAC CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGGAAG 4980
GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCTCC ATCCAGTCTA TTAATTGTTG 5040
CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGCCATTGC 5100
TACAGGCATC GTGGTGTCAC GCTCGTCGTT TGGTATGGCT TCATTCAGCT CCGGTTCCCA 5160
ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG 5220
TCCTCCGATC GTTGTCAGAA GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC 5280
ACTGCATAAT TCTCTTACTG TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA 5340
CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC 5400
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CACTCGTGCA CCCAACTGAT CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC 5580
AAAAACAGGA AGGCAAAATG CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT 5640
ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG 5700
CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA GGGGTTCCGC GCACATTTCC 5760
CCGAAAAGTG CCACCTGACG TCTAAGAAAC CATTATTATC ATGACATTAA CCTATAAAAA 5820
TAGGCGTATC ACGAGGCCCT TTCGTCTCGC GCGTTTCGGT GATGACGGTG AAAACCTCTG 5880
ACACATGCAG CTCCCGGAGA CGGTCACAGC TTGTCTGTAA GCGGATGCCG GGAGCAGACA 5940
AGCCCGTCAG GGCGCGTCAG CGGGTGTTGG CGGGTGTCGG GGCTGGCTTA ACTATGCGGC 6000
ATCAGAGCAG ATTGTACTGA GAGTGCAC 6028

Figure 10. FBdelPMOSAF Sequence 1

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TCGCCATTCA GGCTGCGCAA CTGTTGGGAA GGGCGATCGG TGCGGGCCTC TTCGCTATTA  120
CGCCAGCTGG CGAAAGGGGG ATGTGCTGCA AGGCGATTAA GTTGGGTAAC GCCAGGGTTT  180
TCCCAGTCAC GACGTTGTAA AACGACGGCC AGTGAATTCC GATTAGTTCA ATTTGTTAAA  240
GACAGGATCT CAGTAGTCCA GGCTTTAGTC CTGACTCAAC AATACCACCA GCTAAAACCA  300
CTAGAATACG AGCCACAATA AATAAAAGAT TTTATTTAGT TTCCAGAAAA AGGGGGGAAT  360
GAAAGACCCC ACCAAATTGC TTAGCCTGAT AGCCGCAGTA ACGCCATTTT GCAAGGCATG  420
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GCGCTTCTGC TTCCCGAGCT CTATAAAAGA GCTCACAACC CCTCACTCGG CGCGCCAGTC  780
CTCCGATAGA CTGAGTCGCC CGGGTACCCG TGTATCCAAT AAATCCTCTT GCTGTTGCAT  840
CCGACTCGTG GTCTCGCTGT TCCTTGGGAG GGTCTCCTCA GAGTGATTGA CTACCCGTCT  900
CGGGGGTCTT TCATTTGGGG GCTCGTCCGG GATCTGGAGA CCCCTGCCCA GGGACCACCG  960
ACCCACCACC GGGAGGTAAG CTGGCCAAGA TCTTATATGG GGCACCCCCG CCCCTTGTAA 1020
ACTTCCCTGA CCCTGACATG ACAAGAGTTA CTAACAGCCC CTCTCTCCAA GCTCACTTAC 1080
AGGCTCTCTA CTTAGTCCAG CACGAAGTCT GGAGACCTCT GGCGGCAGCC TACCAAGAAC 1140
AACTGGACCG ACCGGTGGTA CCTCACCCTT ACCGAGTCGG CGACACAGTG TGGGTCCGCC 1200
GACACCAGAC TAAGAACCTA GAACCTCGCT GGAAAGGACC TTACACAGTC CTGCTGACCA 1260
CCCCCACCGC CCTCAAAGTA GACGGCATCG CAGCTTGGAT ACACGCCGCC CACGTGAAGG 1320
CTGCCGACCC CGGGGGTGGA CCATCCTCTA GACTGACATG GCGCGTTCAA CGCTCTCAAA 1380
ACCCCTTAAA AATAAGGTTA ACCCGCGAGG CCCCCTAATC CCCTTAATTC TTCTGATGCT 1440
CAGAGGGGTC AGTACTGCTT CGCCCGGCTC CAGTCCTCAT CAAGTCTATA ATATCACCTG 1500
GGAGGTAACC AATGGAGATC GGGAGACGGT ATGGGCAACT TCTGGCAACC ACCCTCTGTG 1560
GACCTGGTGG CCTGACCTTA CCCCAGATTT ATGTATGTTA GCCCACCATG GACCATCTTA 1620
TTGGGGGCTA GAATATCAAT CCCCTTTTTC TTCTCCCCCG GGGCCCCCTT GTTGCTCAGG 1680
GGGCAGCAGC CCAGGCTGTT CCAGAGACTG CGAAGAACCT TTAACCTCCC TCACCCCTCG 1740
GTGCAACACT GCCTGGAACA GACTCAAGCT AGACCAGACA ACTCATAAAT CAAATGAGGG 1800
ATTTTATGTT TGCCCCGGGC CCCACCGCCC CCGAGAATCC AAGTCATGTG GGGGTCCAGA 1860
CTCCTTCTAC TGTGCCTATT GGGGCTGTGA GACAACCGGT AGAGCTTACT GGAAGCCCTC 1920
CTCATCATGG GATTTCATCA CAGTAAACAA CAATCTCACC TCTGACCAGG CTGTCCAGGT 1980
ATGCAAAGAT AATAAGTGGT GCAACCCCTT AGTTATTCGG TTTACAGACG CCGGGAGACG 2040
GGTTACTTCC TGGACCACAG GACATTACTG GGGCTTACGT TTGTATGTCT CCGGACAAGA 2100
TCCAGGGCTT ACATTTGGGA TCCGACTCAG ATACCAAAAT CTAGGACCCC GCGTCCCAAT 2160
AGGGCCAAAC CCCGTTCTGG CAGACCAACA GCCACTCTCC AAGCCCAAAC CTGTTAAGTC 2220
GCCTTCAGTC ACCAAACCAC CCAGTGGGAC TCCTCTCTCC CCTACCCAAC TTCCACCGGC 2280
GGGAACGGAA AATAGGCTGC TAAACTTAGT AGACGGAGCC TACCAAGCCC TCAACCTCAC 2340
CAGTCCTGAC AAAACCCAAG AGTGCTGGTT GTGTCTAGTA GCGGGACCCC CCTACTACGA 2400
AGGGGTTGCC GTCCTGGGTA CCTACTCCAA CCATACCTCT GCTCCAGCCA ACTGCTCCGT 2460
GGCCTCCCAA CACAAGTTGA CCCTGTCCGA AGTGACCGGA CAGGGACTCT GCATAGGAGC 2520
AGTTCCCAAA ACACATCAGG CCCTATGTAA TACCACCCAG ACAAGCAGTC GAGGGTCCTA 2580
TTATCTAGTT GCCCCTACAG GTACCATGTG GGCTTGTAGT ACCGGGCTTA CTCCATGCAT 2640
CTCCACCACC ATACTGAACC TTACCACTGA TTATTGTGTT CTTGTCGAAC TCTGGCCAAG 2700
AGTCACCTAT CATTCCCCCA GCTATGTTTA CGGCCTGTTT GAGAGATCCA ACCGACACAA 2760
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TGCCGCTGGA ATAGGAACAG GGACTACTGC TCTAATGGCC ACTCAGCAAT TCCAGCAGCT 2880
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GTCTCTCACT TCCCTGTCTG AAGTTGTCCT ACAGAATCGA AGGGGCCTAG ACTTGTTATT 3000
TCTAAAAGAA GGAGGGCTGT GTGCTGCTCT AAAAGAAGAA TGTTGCTTCT ATGCGGACCA 3060
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ACTGTTTGAG TCAACTCAAG GATGGTTTGA GGGACTGTTT AACAGATCCC CTTGGTTTAC 3180
CACCTTGATA TCTACCATTA TGGGACCCCT CATTGTACTC CTAATGATTT TGCTCTTCGG 3240
ACCCTGCATT CTTAATCGAT TAGTTCAATT TGTTAAAGAC AGGATCTCAG TAGTCCAGGC 3300
TTTAGTCCTG ACTCAACAAT ACCACCAGCT AAAGCCTATA GAGTACGAGC CATAGGGCGC 3360
CTAGTGTTGA CAATTAATCA TCGGCATAGT ATACGGCATA GTATAATACG ACTCACTATA 3420
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CCGGAGCGGT CGAGTTCTGG ACCGACCGGC TCGGGTTCTC CCGGGACTTC GTGGAGGACG 3540
ACTTCGCCGG TGTGGTCCGG GACGACGTGA CCCTGTTCAT CAGCGCGGTC CAGGACCAGG 3600
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AGTGGTCGGA GGTCGTGTCC ACGAACTTCC GGGACGCCTC CGGGCCGGCC ATGACCGAGA 3720
TCGGCGAGCA GCCGTGGGGG CGGGAGTTCG CCCTGCGCGA CCCGGCCGGC AACTGCGTGC 3780
ACTTCGTGGC CGAGGAGCAG GACTGANNNN CGGACCGGTC GACTTGTTAA CTTGTTTATT 3840
GCAGCTTATA ATGGTTACAA ATAAAGCAAT AGCATCACAA ATTTCACAAA TAAAGCATTT 3900
TTTTCACTGC ATTCTAGTTG TGGTTTGTCC AAACTCATCA ATGTATCTTA TCATGTCTGG 3960
ATCCAGATCT GGGCCCATGC GGCCGCGGAT CGATNNNNAC ATGTGAGCAA AAGGCCAGCA 4020
AAAGGCCAGG AACCGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC TCCGCCCCCC 4080
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Figure 10. FBdelPMOSAF Sequence 2

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ACGCTGTAGG TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT GTGTGCACGA   4320
ACCCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG AGTCCAACCC   4380
GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA GCAGAGCGAG   4440
GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT ACACTAGAAG   4500
GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA GAGTTGGTAG   4560
CTCTTGATCC GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA   4620
GATTACGCGC AGAAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA CGGGGTCTGA   4680
CGCTCAGTGG AACGAAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT CAAAAAGGAT   4740
CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTTAAA TCAATCTAAA GTATATATGA   4800
GTAAACTTGG TCTGACAGTT ACCAATGCTT AATCAGTGAG GCACCTATCT CAGCGATCTG   4860
TCTATTTCGT TCATCCATAG TTGCCTGACT CCCCGTCGTG TAGATAACTA CGATACGGGA   4920
GGGCTTACCA TCTGGCCCCA GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC   4980
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TTTATCCGCC TCCATCCAGT CTATTAATTG TTGCCGGGAA GCTAGAGTAA GTAGTTCGCC   5100
AGTTAATAGT TTGCGCAACG TTGTTGCCAT TGCTACAGGC ATCGTGGTGT CACGCTCGTC   5160
GTTTGGTATG GCTTCATTCA GCTCCGGTTC CCAACGATCA AGGCGAGTTA CATGATCCCC   5220
CATGTTGTGC AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA GAAGTAAGTT   5280
GGCCGCAGTG TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA CTGTCATGCC   5340
ATCCGTAAGA TGCTTTTCTG TGACTGGTGA GTACTCAACC AAGTCATTCT GAGAATAGTG   5400
TATGCGGCGA CCGAGTTGCT CTTGCCCGGC GTCAATACGG GATAATACCG CGCCACATAG   5460
CAGAACTTTA AAAGTGCTCA TCATTGGAAA ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT   5520
CTTACCGCTG TTGAGATCCA GTTCGATGTA ACCCACTCGT GCACCCAACT GATCTTCAGC   5580
ATCTTTTACT TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA ATGCCGCAAA   5640
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TTGAAGCATT TATCAGGGTT ATTGTCTCAT GAGCGGATAC ATATTTGAAT GTATTTAGAA   5760
AAATAAACAA ATAGGGGTTC CGCGCACATT TCCCCGAAAA GTGCCACCTG ACGTCTAAGA   5820
AACCATTATT ATCATGACAT TAACCTATAA AAATAGGCGT ATCACGAGGC CCTTTCGTCT   5880
CGCGCGTTTC GGTGATGACG GTGAAAACCT CTGACACATG CAGCTCCCGG AGACGGTCAC   5940
AGCTTGTCTG TAAGCGGATG CCGGGAGCAG ACAAGCCCGT CAGGGCGCGT CAGCGGGTGT   6000
TGGCGGGTGT CGGGGCTGGC TTAACTATGC GGCATCAGAG CAGATTGTAC TGAGAGTGCA   6060
C                                                                   6061
```

Figure 11. FBdelPGASAF Sequence 1

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CGCCAGCTGG CGAAAGGGGG ATGTGCTGCA AGGCGATTAA GTTGGGTAAC GCCAGGGTTT 180
TCCCAGTCAC GACGTTGTAA AACGACGGCC AGTGAATTCC GATTAGTTCA ATTTGTTAAA 240
GACAGGATCT CAGTAGTCCA GGCTTTAGTC CTGACTCAAC AATACCACCA GCTAAAACCA 300
CTAGAATACG AGCCACAATA AATAAAAGAT TTTATTTAGT TTCCAGAAAA AGGGGGGAAT 360
GAAAGACCCC ACCAAATTGC TTAGCCTGAT AGCCGCAGTA ACGCCATTTT GCAAGGCATG 420
GAAAAATACC AAACCAAGAA TAGAGAAGTT CAGATCAAGG GCGGGTACAC GAAAACAGCT 480
AACGTTGGGC CAAACAGGAT ATCTGCGGTG AGCAGTTTCG GCCCCGGCCC GGGGCCAAGA 540
ACAGATGGTC ACCGCGGTTC GGCCCCGGCC CGGGGCCAAG AACAGATGGT CCCCAGATAT 600
GGCCCAACCC TCAGCAGTTT CTTAAGACCC ATCAGATGTT TCCAGGCTCC CCCAAGGACC 660
TGAAATGACC CTGTGCCTTA TTTGAATTAA CCAATCAGCC TGCTTCTCGC TTCTGTTCGC 720
GCGCTTCTGC TTCCCGAGCT CTATAAAAGA GCTCACAACC CCTCACTCGG CGCGCCAGTC 780
CTCCGATAGA CTGAGTCGCC CGGGTACCCG TGTATCCAAT AAATCCTCTT GCTGTTGCAT 840
CCGACTCGTG GTCTCGCTGT TCCTTGGGAG GGTCTCCTCA GAGTGATTGA CTACCCGTCT 900
CGGGGGTCTT TCATTTGGGG GCTCGTCCGG GATCTGGAGA CCCCTGCCCA GGGACCACCG 960
ACCCACCACC GGGAGGTAAG CTGGCCAAGA TCCCTAAGGT ACTCGGGTCA GACAATGGCC 1020
CGGCCTTTGT TGCTCAGGTA AGTCAGGGAC TGGCCACTCA ACTGGGGATA AATTGGAAGT 1080
TACATTGTGC GTATAGACCC CAGAGCTCAG GTCAGGTAGA AAGAATGAAC AGAACAATTA 1140
AAGAGACCTT GACCAAATTA GCCTTAGAGA CCGGTGGAAA AGACTGGGTG ACCCTCCTTC 1200
CCTTAGCGCT GCTTAGGGCC AGGAATACCC CTGGCCGGTT TGGTTTAACT CCTTATGAAA 1260
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GATTTCTCCC TGTCTTATTT ACTCACTTAA AGGCTTTAGA AATTGTAAGG ACCCAAATCT 1380
GGGACCAGAT CAAAGAGGTG TATAAGCCTG GTACCGTAAC AATCCCTCAC CCGTTCCAGG 1440
TCGGGGATCA AGTGCTTGTC AGACGCCATC GACCCAGCAG CCTTGAGCCT CGGTGGAAAG 1500
GCCCATACCT GGTGTTGCTG ACTACCCCGA CCGCGGTAAA AGTCGATGGT ATTGCTGCCT 1560
GGGTCCATGC TTCTCACCTC AAACCTGCAC CACCTTCGGC ACCAGATGAG TCCTGGGAGC 1620
TGGAAAAAGAC TGATCATCCT CTTAAGCTGC GTATTCGGCG GCGGCGGGAC GAGTCTGCAA 1680
AATAAGAACC CCCACCAGCC CATGACCCTC ACTTGGCAGG TACTGTCCCA AACTGGAGAC 1740
GTTGTCTGGG ATACAAAGGC AGTCCAGCCC CCTTGGACTT GGTGGCCCAC ACTTAAACCT 1800
GATGTATGTG CCTTGGCGGC TAGTCTTGAG TCCTGGGATA TCCCGGGAAC CGATGTCTCG 1860
TCCTCTAAAC GAGTCAGACC TCCGGACTCA GACTATACTG CCGCTTATAA GCAAATCACC 1920
TGGGGAGCCA TAGGGTGCAG CTACCCTCGG GCTAGGACTA GAATGGCAAG CTCTACCTTC 1980
TACGTATGTC CCCGGGATGG CCGGACCCTT TCAGAAGCTA GAAGGTGCGG GGGGCTAGAA 2040
TCCCTATACT GTAAAGAATG GGATTGTGAG ACCACGGGGA CCGGTTATTG GCTATCTAAA 2100
TCCTCAAAAG ACCTCATAAC TGTAAAATGG GACCAAAATA GCGAATGGAC TCAAAAATTT 2160
CAACAGTGTC ACCAGACCGG CTGGTGTAAC CCCCTTAAAA TAGATTTCAC AGACAAAGGA 2220
AAATTATCCA AGGACTGGAT AACGGGAAAA ACCTGGGGAT TAAGATTCTA TGTGTCTGGA 2280
CATCCAGGCG TACAGTTCAC CATTCGCTTA AAAATCACCA ACATGCCAGC TGTGGCAGTA 2340
GGTCCTGACC TCGTCCTTGT GGAACAAGGA CCTCCTAGAA CGTCCCTCGC TCTCCCACCT 2400
CCTCTTCCCC CAAGGGAAGC GCCACCGCCA TCTCTCCCCG ACTCTAACTC CACAGCCCTG 2460
GCGACTAGTG CACAAACTCC CACGGTGAGA AAAACAATTG TTACCCTAAA CACTCCGCCT 2520
CCCACCACAG GCGACAGACT TTTTGATCTT GTGCAGGGGG CCTTCCTAAC CTTAAATGCT 2580
ACCAACCCAG GGGCCACTGA GTCTTGCTGG CTTTGTTTGG CCATGGGCCC CCCTTATTAT 2640
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GGGACCCAAG GAAAGCTCAC CCTCACTGAG GTCTCAGGAC ACGGGTTGTG CATAGGAAAG 2760
GTGCCCTTTA CCCATCAGCA TCTCTGCAAT CAGACCCTAT CCATCAATTC CTCCGGAGAC 2820
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TGCCTCTCCA CCTCAGTTTT TAATCAGACT AGAGATTTCT GTATCCAGGT CCAGCTGATT 2940
CCTCGCATCT ATTACTATCC TGAAGAAGTT TTGTTACAGG CCTATGACAA TTCTCACCCC 3000
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CTGACAAGCC TCCAGATCGC CATAGATGCT GACCTCCGGG CCCTCCAAGA CTCAGTCAGC 3180
AAGTTAGAGG ACTCACTGAC TTCCCTGTCC GAGGTAGTGC TCCAAAATAG GAGAGGCCTT 3240
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AAAAGACAGT TAGAGCGCCA GAAAAGCCAA AACTGGTATG AAGGATGGTT CAATAACTCC 3420
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CTCATCCTCG GGCCATGCAT CATCAATCGA TTAGTTCAAT TTGTTAAAGA CAGGATCTCA 3540
GTAGTCCAGG CTTTAGTCCT GACTCAACAA TACCACCAGC TAAAGCCTAT AGAGTACGAG 3600
CCATAGGGCG CCTAGTGTTG ACAATTAATC ATCGGCATAG TATACGGCAT AGTATAATAC 3660
GACTCACTAT AGGAGGGCCA CCATGGCCAA GTTGACCAGT GCCGTTCCGG TGCTCACCGC 3720
GCGCGACGTC GCCGGAGCGG TCGAGTTCTG GACCGACCGG CTCGGGTTCT CCCGGGACTT 3780
CGTGGAGGAC GACTTCGCCG GTGTGGTCCG GGACGACGTG ACCCTGTTCA TCAGCGCGGT 3840
CCAGGACCAG GTGGTGCCGG ACAACACCCT GGCCTGGGTG TGGGTGCGCG GCCTGGACGA 3900
GCTGTACGCC GAGTGGTCGG AGGTCGTGTC CACGAACTTC CGGGACGCCT CCGGGCCGGC 3960
CATGACCGAG ATCGGCGAGC AGCCGTGGGG GCGGGAGTTC GCCCTGCGCG ACCCGGCCGG 4020
CAACTGCGTG CACTTCGTGG CCGAGGAGCA GGACTGANNN NCGGACCGGT CGACTTGTTA 4080
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Figure 11. FBdelPGASAF Sequence 2

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AAAGGCCAGC AAAAGGCCAG GAACCGTAAA AAGGCCGCGT TGCTGGCGTT TTTCCATAGG   4320
CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA GTCAGAGGTG GCGAAACCCG   4380
ACAGGACTAT AAAGATACCA GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT   4440
CCGACCCTGC CGCTTACCGG ATACCTGTCC GCCTTTCTCC CTTCGGGAAG CGTGGCGCTT   4500
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TGTGTGCACG AACCCCCCGT TCAGCCCGAC CGCTGCGCCT TATCCGGTAA CTATCGTCTT   4620
GAGTCCAACC CGGTAAGACA CGACTTATCG CCACTGGCAG CAGCCACTGG TAACAGGATT   4680
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TCAAAAAGGA TCTTCACCTA GATCCTTTTA AATTAAAAAT GAAGTTTTAA ATCAATCTAA   5040
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TCAGCGATCT GTCTATTTCG TTCATCCATA GTTGCCTGAC TCCCCGTCGT GTAGATAACT   5160
ACGATACGGG AGGGCTTACC ATCTGGCCCC AGTGCTGCAA TGATACCGCG AGACCCACGC   5220
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TGATCTTCAG CATCTTTTAC TTTCACCAGC GTTTCTGGGT GAGCAAAAAC AGGAAGGCAA   5880
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TTTCAATATT ATTGAAGCAT TTATCAGGGT TATTGTCTCA TGAGCGGATA CATATTTGAA   6000
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GACGTCTAAG AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGCG TATCACGAGG   6120
CCCTTTCGTC TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG   6180
GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG   6240
TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA   6300
CTGAGAGTGC AC                                                       6312
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Figure 12. FBdelPRDSAF Sequence

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CATATGCGGT GTGAAATACC GCACAGATGC GTAAGGAGAA AATACCGCAT CAGGCGCCAT   60
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TCCCAGTCAC GACGTTGTAA AACGACGGCC AGTGAATTCC GATTAGTTCA ATTTGTTAAA  240
GACAGGATCT CAGTAGTCCA GGCTTTAGTC CTGACTCAAC AATACCACCA GCTAAAACCA  300
CTAGAATACG AGCCACAATA AATAAAAGAT TTTATTTAGT TTCCAGAAAA AGGGGGGAAT  360
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GGCCCAACCC TCAGCAGTTT CTTAAGACCC ATCAGATGTT TCCAGGCTCC CCCAAGGACC  660
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ACCTTGTCCC TGTACTAACC CAAAATGAAA CTCCCAACAG GAATGGTCAT TTTATGTAGC 1500
CTAATAATAG TTCGGGCAGG GTTTGACGAC CCCCGCAAGG CTATCGCATT AGTACAAAAA 1560
CAACATGGTA AACCATGCGA ATGCAGCGGA GGGCAGGTAT CCGAGGCCCC ACCGAACTCC 1620
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ACTTTCCAGG ACTCGATGCA CAGTTCTTGT TATACTGAAT ACCGGCAATG CAGGCGAATT 1800
AATAAGACAT ACTACACGGC CACCTTGCTT AAAATACGGT CTGGGAGCCT CAACGAGGTA 1860
CAGATATTAC AAAACCCCAA TCAGCTCCTA CAGTCCCCTT GTAGGGGCTC TATAAATCAG 1920
CCCGTTTGCT GGAGTGCCAC AGCCCCCATC CATATCTCCG ATGGTGGAGG ACCCCTCGAT 1980
ACTAAGAGAG TGTGGACAGT CCAAAAAAGG CTAGAACAAA TTCATAAGGC TATGACTCCT 2040
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CGGACTTTTG ATATCCTGAA TACCACTTTT AGGTTACTCC AGATGTCCAA TTTTAGCCTT 2160
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CTGGCAACCA ACCCTCTCTG GACCGGGCTG CAGGGCTTTC TTCCGTACCT CCTACCTCTC 3000
CTGGGACCCC TACTCACCCT CCTACTCATA CTAACCATTG GGCCATGCGT TTTCAGTCGC 3060
CTCATGGCCT TCATTAATGA TAGACTTAAT GTTGTACATG CCATGGTGCT GGCCCAGCAA 3120
TACCAAGCAC TCAAAGCTGA GGAAGAAGCT CAGGATTGAG GCGCCTAGTG TTGACAATTA 3180
ATCATCGGCA TAGTATACGG CATAGTATAA TACGACTCAC TATAGGAGGG CCACCATGGC 3240
CAAGTTGACC AGTGCCGTTC CGGTGCTCAC CGCGCGCGAC GTCGCCGGAG CGGTCGAGTT 3300
CTGGACCGAC CGGCTCGGGT TCTCCCGGGA CTTCGTGGAG GACGACTTCG CCGGTGTGGT 3360
CCGGGACGAC GTGACCCTGT TCATCAGCGC GGTCCAGGAC CAGGTGGTGC CGGACAACAC 3420
CCTGGCCTGG GTGTGGGTGC GCGGCCTGGA CGAGCTGTAC GCCGAGTGGT CGGAGGTCGT 3480
GTCCACGAAC TTCCGGGACG CCTCCGGGCC GGCCATGACC GAGATCGGCG AGCAGCCGTG 3540
GGGGCGGGAG TTCGCCCTGC GCGACCCGGC CGGCAACTGC GTGCACTTCG TGGCCGAGGA 3600
GCAGGACTGA NNNNCGGACC GGTCGACTTG TTAACTTGTT TATTGCAGCT TATAATGGTT 3660
ACAAATAAAG CAATAGCATC ACAAATTTCA CAAATAAAGC ATTTTTTTCA CTGCATTCTA 3720
GTTGTGGTTT GTCCAAACTC ATCAATGTAT CTTATCATGT CTGGATCCAG ATCTGGGCCC 3780
ATGCGGCCGC GGATCGATNN NNACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT 3840
AAAAAGGCCG CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA 3900
AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT 3960
CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG 4020
TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG CTTTCTCAAT GCTCACGCTG TAGGTATCTC 4080
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Figure 12. FBdelPRDSAF Sequence 2

AGTTCGGTGT AGGTCGTTCG CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC 4140
GACCGCTGCG CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA 4200
TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT 4260
ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA GAAGGACAGT ATTTGGTATC 4320
TGCGCTCTGC TGAAGCCAGT TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA 4380
CAAACCACCG CTGGTAGCGG TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA 4440
AAAGGATCTC AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA 4500
AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT 4560
TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC 4620
AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC 4680
ATAGTTGCCT GACTCCCCGT CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC 4740
CCCAGTGCTG CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA 4800
AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC 4860
CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC 4920
AACGTTGTTG CCATTGCTAC AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA 4980
TTCAGCTCCG GTTCCCAACG ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA 5040
GCGGTTAGCT CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA 5100
CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT 5160
TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT 5220
TGCTCTTGCC CGGCGTCAAT ACGGGATAAT ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG 5280
CTCATCATTG GAAAACGTTC TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA 5340
TCCAGTTCGA TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC 5400
AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG 5460
ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT ATTATTGAAG CATTTATCAG 5520
GGTTATTGTC TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA ACAAATAGGG 5580
GTTCCGCGCA CATTTCCCCG AAAAGTGCCA CCTGACGTCT AAGAAACCAT TATTATCATG 5640
ACATTAACCT ATAAAAATAG GCGTATCACG AGGCCCTTTC GTCTCGCGCG TTTCGGTGAT 5700
GACGGTGAAA ACCTCTGACA CATGCAGCTC CCGGAGACGG TCACAGCTTG TCTGTAAGCG 5760
GATGCCGGGA GCAGACAAGC CCGTCAGGGC GCGTCAGCGG GTGTTGGCGG GTGTCGGGGC 5820
TGGCTTAACT ATGCGGCATC AGAGCAGATT GTACTGAGAG TGCAC 5865

Figure 13. hCMV10A1 Sequence

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AGATCTCCCG ATCCCCTATG GTCGACTCTC AGTACAATCT GCTCTGATGC CGCATAGTTA     60
AGCCAGTATC TGCTCCCTGC TTGTGTGTTG GAGGTCGCTG AGTAGTGCGC GAGCAAAATT    120
TAAGCTACAA CAAGGCAAGG CTTGACCGAC AATTGCATGA AGAATCTGCT TAGGGTTAGG    180
CGTTTTGCGC TGCTTCGCGA TGTACGGGCC AGATATACGC GTTGACATTG ATTATTGACT    240
AGTTATTAAT AGTAATCAAT TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC    300
GTTACATAAC TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG    360
ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA TTGACGTCAA    420
TGGGTGGACT ATTTACGGTA AACTGCCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA    480
AGTACGCCCC CTATTGACGT CAATGACGGT AAATGGCCCG CCTGGCATTA TGCCCAGTAC    540
ATGACCTTAT GGGACTTTCC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC    600
ATGGTGATGC GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA    660
TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA AAATCAACGG    720
GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA    780
CGGTGGGAGG TCTATATAAG CAGAGCTCTC TGGCTAACTA GAGAACCCAC TGCTTAACTG    840
GCTTATCGAA ATGTCGACTG AGAACTTCAG GGTGAGTTTG GGGACCCTTG ATTGTTCTTT    900
CTTTTTCGCT ATTGTAAAAT TCATGTTATA TGGAGGGGGC AAAGTTTTCA GGGTGTTGTT    960
TAGAATGGGA AGATGTCCCT TGTATCACCA TGGACCCTCA TGATAATTTT GTTTCTTTCA   1020
CTTTCTACTC TGTTGACAAC CATTGTCTCC TCTTATTTTC TTTTCATTTT CTGTAACTTT   1080
TTCGTTAAAC TTTAGCTTGC ATTTGTAACG AATTTTTAAA TTCACTTTTG TTTATTTGTC   1140
AGATTGTAAG TACTTTCTCT AATCACTTTT TTTTCAAGGC AATCAGGGTA TATTATATTG   1200
TACTTCAGCA CAGTTTTAGA GAACAATTGT TATAATTAAA TGATAAGGTA GAATATTTCT   1260
GCATATAAAT TCTGGCTGGC GTGGAAATAT TCTTATTGGT AGAAACAACT ACATCCTGGT   1320
CATCATCCTG CCTTTCTCTT TATGGTTACA ATGATATACA CTGTTTGAGA TGAGGATAAA   1380
ATACTCTGAG TCCAAACCGG GCCCCTCTGC TAACCATGTT CATGCCTTCT TCTTTTTCCT   1440
ACAGCTCCTG GGCAACGTGC TGGTTGTTGT GCTGTCTCAT CATTTTGGCA AGGATCGGCC   1500
GGAACAGCAT CAGGACCGAC ATGGAAGGTC CAGCGTTCTC AAAACCCCTT AAAGATAAGA   1560
TTAACCCGTG GAAGTCCTTA ATGGTCATGG GGGTCTATTT AAGAGTAGGG ATGGCAGAGA   1620
GCCCCCATCA GGTCTTTAAT GTAACCTGGA GAGTCACCAA CCTGATGACT GGGCGTACCG   1680
CCAATGCCAC CTCCCTTTTA GGAACTGTAC AAGATGCCTT CCCAAGATTA TATTTTGATC   1740
TATGTGATCT GGTCGGAGAA GAGTGGGACC CTTCAGACCA GGAACCATAT GTCGGGTATG   1800
GCTGCAAATA CCCCGGAGGG AGAAAGCGGA CCCGGACTTT TGACTTTTAC GTGTGCCCTG   1860
GGCATACCGT AAAATCGGGG TGTGGGGGGC CAAGAGAGGG CTACTGTGGT GAATGGGGTT   1920
GTGAAACCAC CGGACAGGCT TACTGGAAGC CCACATCATC ATGGGACCTA ATCTCCCTTA   1980
AGCGCGGTAA CACCCCCTGG GACACGGGAT GCTCCAAAAT GGCTTGTGGC CCCTGCTACG   2040
ACCTCTCCAA AGTATCCAAT TCCTTCCAAG GGGCTACTCG AGGGGGCAGA TGCAACCCTC   2100
TAGTCCTAGA ATTCACTGAT GCAGGAAAAA AGGCTAATTG GGACGGGCCC AAATCGTGGG   2160
GACTGAGACT GTACCGGACA GGAACAGATC CTATTACCAT GTTCTCCCTG ACCCGCCAGG   2220
TCCTCAATAT AGGGCCCCGC ATCCCCATTG GGCCTAATCC CGTGATCACT GGTCAACTAC   2280
CCCCCTCCCG ACCCGTGCAG ATCAGGCTCC CCAGGCCTCC TCAGCCTCCT CCTACAGGCG   2340
CAGCCTCTAT AGTCCCTGAG ACTGCCCCAC CTTCTCAACA ACCTGGGACG GGAGACAGGC   2400
TGCTAAACCT GGTAGAAGGA GCCTATCAGG CGCTTAACCT CACCAATCCC GACAAGACCC   2460
AAGAATGTTG GCTGTGCTTA GTGTCGGGAC CTCCTTATTA CGAAGGAGTA GCGGTCGTGG   2520
GCACTTATAC CAATCATTCT ACCGCCCCGG CCAGCTGTAC GGCCACTTCC CAACATAAGC   2580
TTACCCTATC TGAAGTGACA GGACAGGGCC TATGCATGGG AGCACTACCT AAAACTCACC   2640
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CTGGAACAAT GTGGGCTTGT AGCACTGGAT TGACTCCCTG CTTGTCCACC ACGATGCTCA   2760
ATCTAACCAC AGACTATTGT GTATTAGTTG AGCTCTGGCC CAGAATAATT TACCACTCCC   2820
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TGACCCTGGC CCTTCTGCTA GGAGGATTAA CCATGGGAGG GATTGCAGCT GGAATAGGGA   2940
CGGGGACCAC TGCCCTAATC AAAACCCAGC AGTTTGAGCA GCTTCACGCC GCTATCCAGA   3000
CAGACCTCAA CGAAGTCGAA AAATCAATTA CCAACCTAGA AAAGTCACTG ACCTCGTTGT   3060
CTGAAGTAGT CCTACAGAAC CGAAGAGGCC TAGATTTGCT CTTCCTAAAA GAGGGAGGTC   3120
TCTGCGCAGC CCTAAAAGAA GAATGTTGTT TTTATGCAGA CCACACGGGA CTAGTGAGAG   3180
ACAGCATGGC CAAACTAAGG GAAAGGCTTA ATCAGAGACA AAAACTATTT GAGTCAGGCC   3240
AAGGTTGGTT CGAAGGGCAG TTTAATAGAT CCCCCTGGTT TACCACCTTA ATCTCCACCA   3300
TCATGGGACC TCTAATAGTA CTCTTACTGA TCTTACTCTT TGGACCCTGC ATTCTCAATC   3360
GATTAGTTCA ATTTGTTAAA GACAGGATCT CAGTAGTCCA GGCTTTAGTC CTGACTCAAC   3420
AATACCACCA GCTAAAGCCT ATAGAGTACG AGCCATAGGG CGCCTAGTGT TGACAATTAA   3480
TCATCGGCAT AGTATACGGC ATAGTATAAT ACGACTCACT ATAGGAGGGC CACCATGGCC   3540
AAGTTGACCA GTGCCGTTCC GGTGCTCACC GCGCGCGACG TCGCCGGAGC GGTCGAGTTC   3600
TGGACCGACC GGCTCGGGTT CTCCCGGGAC TTCGTGGAGG ACGACTTCGC CGGTGTGGTC   3660
CGGGACGACG TGACCCTGTT CATCAGCGCG GTCCAGGACC AGGTGGTGCC GGACAACACC   3720
CTGGCCTGGG TGTGGGTGCG CGGCCTGGAC GAGCTGTACG CCGAGTGGTC GGAGGTCGTG   3780
TCCACGAACT TCCGGGACGC CTCCGGGCCG GCCATGACCG AGATCGGCGA GCAGCCGTGG   3840
GGGCGGGAGT TCGCCCTGCG CGACCCGGCC GGCAACTGCG TGCACTTCGT GGCCGAGGAG   3900
CAGGACTGAN NNNCGGACCG GTCGA                                         3925
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/02061

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/10 C12N15/67

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF VIROLOGY 69 (7). 1995. 4086-4094. ISSN: 0022-538X, July 1995, XP002023654 LUUKKONEN B G M ET AL: "Efficiency of reinitiation of translation on human immunodeficiency virus type 1 mRNAs is determined by the length of the upstream open reading frame and by intercistronic distance." see the whole document ---	1-29
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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 January 1997	12.02.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Hornig, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

International Application No
PCT/GB 96/02061

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		CA-A- 2162056	10-11-94
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		JP-T- 8511156	26-11-96

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